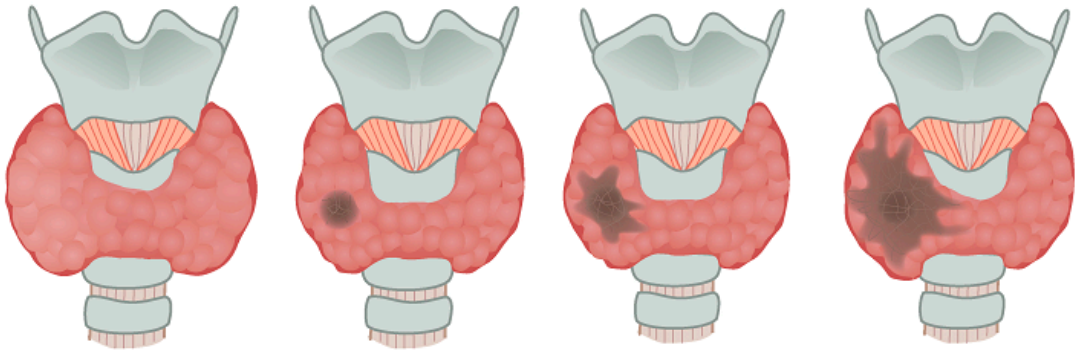
FOLLICULAR THYROID TUMOURS - MARKERS OF NEOPLASIA, MALIGNANCY AND PROGNOSIS

Annukka Heikkilä



Helsinki 2013

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To my family

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1 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by their Roman numerals:

I Heikkilä A, Siironen P, Hagström J, Heiskanen I, Sankila R, Louhimo J, Haglund C*, Arola J*. Follicular thyroid neoplasm: clinicopathological features suggesting malignancy. APMIS. 2010 Nov; 118(11): 846-54. * equal contribution

II Heikkilä A, Fermér C, Hagström J, Louhimo J, Mäenpää H, Siironen P, Heiskanen I, Nilsson O, Arola J*, Haglund C*. HES 5 – a novel stem cell marker differentiating between neoplastic lesions in follicular thyroid neoplasms. In manuscript. * equal contribution.

III Heikkilä A, Hagström J, Mäenpää H, Louhimo J, Siironen P, Heiskanen I, Haglund C*, Arola J*. Loss of estrogen receptor β expression in follicular thyroid carcinoma predicts poor outcome. Thyroid. 2013 Apr; 23(4): 456-65. * equal contribution.

IV Hagström J*, Heikkilä A*, Siironen P, Louhimo J, Heiskanen I, Mäenpää H, Arola J**, Haglund C**. TLR-4 expression and decrease in chronic inflammation: indicators of aggressive follicular thyroid carcinoma. Journal of Clinical Pathology. 2012 Apr; 65(4): 333-8. * shared first authorship, ** equal contribution

2 ABBREVIATIONS

ABC	avidin-biotin complex
AEC	3-amino-9-ethylcarbazole
ATA	American Thyroid Association
ATC	anaplastic/undifferentiated thyroid carcinoma
AUC	area-under-the curve
AUS	atypia of undetermined significance
BRAF	b-raf murine sarcoma viral oncogene homologue B1
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CD31/PECAM1	platelet endothelial cell adhesion molecule-1
CDK	cyclin-dependent kinase
CI	confidence interval
CK-19	cytokeratin-19
COX-2	cyclooxygenase 2
CpG	cytosine-phosphate-guanosine
CSC	cancer stem cell
CT	computed tomography
DAB	3,3'-diaminobenzidine
DPP IV	dipeptidyl peptidase 4
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ERα / β	estrogen receptor α/β
FFTC	familial follicular thyroid cancer
FLUS	follicular lesion of undetermined significance
FNAB	fine-needle aspiration biopsy
FNMTC	familial nonmedullary thyroid cancer
FTA	follicular thyroid adenoma
FTC	follicular thyroid carcinoma
FVPTC	follicular variant of papillary thyroid carcinoma
HBME-1	Hector Battifora mesothelial cell 1
HE	haematoxylin-eosin
HES5	human embryonic stem cell 5
HGF	hepatocyte growth factor
HPT	hypothalamus-pituitary-thyroid
HRP	horseradish peroxidase
HUCH	Helsinki University Central Hospital
IgG	immunoglobulin G
IL-8	interleukin 8
IMP-3	insulin-like growth factor mRNA binding protein 3
LOH	loss of heterozygosity
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAP	mitogen-activated protein
MEN2	multiple endocrine neoplasia 2
MIB-1	mind bomb E3 ubiquitin protein ligase 1
MIG	monocyte-induced interferon gamma
miRNA	microRNA
MMP	matrix metalloproteinase
MNG	multinodular goitre

mRNA	messenger ribonucleic acid
MTC	medullary thyroid carcinoma
NF-κB	nuclear factor kappa in B cells
NRTK1	neurotrophic tyrosine kinase, receptor, type 1
p27	protein 27
p53	protein 53
pAb	polyclonal antibody
PAMP	pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PAX8-PPARγ	paired box gene 8 - peroxisome proliferator-activated receptor γ
PBDE	polybrominated diphenyl ether
PBS	phosphate-buffered saline
PDFTC	poorly differentiated follicular thyroid carcinoma
PDTC	poorly differentiated thyroid carcinoma
PET	positron emission tomography
PI	proliferative index
PI3K-AKT	phosphatidylinositol-3-kinase-protein kinase B pathway
PI3KCA	phosphatidylinositol 3-kinase, catalytic, alpha polypeptide
PRKAR1A	protein kinase, cyclic adenosine monophosphate-dependent, regulatory, type 1, alpha
PT	pretreatment
PTC	papillary thyroid carcinoma
PTEN	phosphatase and tensin homologue
RAS	rat sarcoma
RB	retinoblastoma
RET	rearranged during transfection proto-oncogene
rhTSH	recombinant human thyrotropin
RNA	ribonucleic acid
ROC	receiver-operating characteristic
SEER	Surveillance Epidemiology and End Results
SPSS	Statistical Package for the Social Sciences
T3	triiodothyroxine
T4	thyroxin/tetraiodothyronine
TAM	tumour-associated macrophage
TGFR	transforming growth factor receptor
TGFβ	transforming growth factor β
TLR	toll-like receptor
TMA	tissue microarray
TNM	tumour-node-metastasis classification
TP53/p53	tumour protein 53
TPO	thyroid peroxidase
TRH	thyrotropin-releasing hormone
Tris-Hcl	Tris(hydroxymethyl)aminomethane hydrochloride
TSH	thyroid-stimulating hormone/thyrotropin
TTF-1	thyroid transcription factor 1
UICC	Union of International Cancer Control
US	ultrasonography
WDFTC	well-differentiated follicular thyroid carcinoma
WDTC	well-differentiated thyroid carcinoma
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WRN	Werner syndrome, RecQ helicaselike
WWF	World Wildlife Fund

3 ABSTRACT

Follicular thyroid carcinoma (FTC) is the second most common malignancy of the thyroid gland, with predisposing genetic alterations such as rat sarcoma (RAS) mutation and paired box gene 8-peroxisome proliferator-activated receptor γ (PAX-PPARγ) alteration, as well as suggested risk factors such as iodine insufficiency and female gender. Distinguishing FTC from the most common neoplasm of the thyroid, follicular thyroid adenoma (FTA), or even from a non-neoplastic goitrous nodule, is often impossible preoperatively, leading to unnecessary surgery and exposing patients to surgical complications.

In this study, 127 follicular thyroid neoplasia patients (83 FTAs and 44 FTCs) treated at Helsinki University Central Hospital (HUCH) in Finland between 1990 and 2009 were examined to find methods for differential diagnosis between follicular thyroid lesions. Tissue markers were investigated by immunohistochemistry in follicular neoplasms and non-neoplastic control tissues, and were correlated with clinical parameters, such as with metastatic disease and survival. Additionally, cancer registry data from the HUCH region were gathered, concerning the diminishing incidence of FTC accompanied by an increase in the incidence of papillary thyroid carcinoma.

Carcinomas were reanalysed according to the new World Health Organization classification of endocrine tumours, in which a new tumour entity, poorly differentiated carcinoma of the thyroid, was introduced. Markers with possible clinical utility were found; e.g. in an attempt to differentiate between non-neoplastic and neoplastic follicular lesions of the thyroid (HES5) as well as between FTA and FTC (MIB-1, Cyclin D1, TLR-2, ERβ), a marker with prognostic value in carcinomas (ERβ), as well as a marker correlating with the presence of metastatic disease (TLR-4).

These results aid in the challenging field of diagnostics in follicular thyroid lesions. Measuring the expression of HES5 may help in differentiating between neoplastic and non-neoplastic follicular thyroid lesions. Markers, such as MIB-1 and ERβ, are partly able to differentiate between benign and malignant follicular thyroid neoplasias, whereas ERβ and TLR-4 have prognostic value in FTC.

4 INTRODUCTION

In Finland, approximately 350–400 new thyroid cancer cases are diagnosed yearly (1). The most common thyroid cancer type is papillary thyroid carcinoma (PTC), a neoplasia showing increasing incidence, whereas follicular thyroid carcinoma (FTC) is the second most common type, with decreasing incidence. Both subtypes are included in the group of well-differentiated thyroid carcinomas (WDTCs) with excellent 5-year survivals of over 95% and 85% respectively (2). The most recent classification of the World Health Organization (WHO) of Endocrine Organs was introduced in 2004 (2), in which a new thyroid tumour entity called poorly differentiated thyroid carcinoma (PDTC) was introduced and categorized between WDTC and anaplastic thyroid carcinoma (ATC) according to behaviour and morphology. Additionally, the WHO has distinguished the follicular variant of PTC (FVPTC) from FTC resulting in a reciprocal decrease in incidence of FTC.

The major risk factors for thyroid carcinoma include ionizing radiation, both therapeutic and accidental, deficiency in dietary iodine, lymphocytic thyroiditis or goitre, certain hereditary conditions such as familial adenomatous polyposis coli in PTC and Cowden syndrome in both FTC and PTC, female gender, older age and diabetes (3,4). PTC especially is linked with ionizing radiation more often than other carcinoma subtypes and women are known to be more susceptible to it (5). The worst historical nuclear power accident in the world in Chernobyl, Ukraine in 1986 increase the prevalence of thyroid carcinomas at least 10-fold. This was especially seen in young children in Belarus, with the first cases arising in 1990. Children under the age of 5 years were particularly prone to develop cancer, especially PTC (6,7).

Malignant thyroid nodules are often asymptomatic, but may present as bulging of the neck and can even cause hoarseness, due to disturbance in laryngeal nerve function. The gold standard for diagnostics of thyroid nodules includes ultrasound (US) imaging combined with fine-needle aspiration biopsy (FNAB). Diagnosis of thyroid nodules is challenging, since cytology is often unable to differentiate between neoplastic and non-neoplastic thyroid nodules. The varying criteria of thyroid neoplasias used worldwide further increases the challenges in the diagnosis of thyroid nodules. In Finland alone, major differences in the incidence numbers of thyroid cancers occur. For example, in the Helsinki University Central Hospital (HUCH) region (1 million residents), about 25 new cancer cases are diagnosed yearly, compared to about 16 new cases yearly in the Oulu University Central Hospital region (0.2 million residents) (I), (8).

Follicular thyroid adenoma (FTA) is the most common neoplasia of the thyroid, and it is indistinguishable from FTCs at cytology. Differential diagnosis between them is made at histology by demonstration of invasive growth through the tumour capsule or to vessels. Occasionally, even non-neoplastic nodules are indistinguishable from neoplastic lesions. This leads to diagnostic surgery, causing unnecessary morbidity and rising healthcare

costs. Many markers have been sought to aid in thyroid diagnostics, but the National Cancer Institute has so far not been able to offer any solution to this diagnostic problem, either by immunocytochemistry, immunohistochemistry or molecular techniques (9,10). This translational thesis on follicular thyroid neoplasia focuses on markers for differential diagnosis between neoplastic and non-neoplastic lesions, as well as between benign and malignant follicular neoplasias.

5 REVIEW OF THE LITERATURE

5.1 Normal thyroid gland

The adult thyroid gland is a bilobated organ weighing from 15 to 25 g, located in the neck in front of the larynx and trachea. The two lobes are connected by the isthmus (Figure 1). The gland is surrounded by a thin fibrous capsule and is divided into lobules. The parathyroid glands are located posterior to the thyroid next to the recurrent laryngeal nerves, which run between the trachea and oesophagus. The blood supply to the thyroid comes from the superior thyroid arteries, which branch from the external carotid arteries, and the inferior thyroid arteries, which branch from the subclavian arteries. The nerves running to the thyroid come from the superior and middle cervical sympathetic ganglia (11-13).

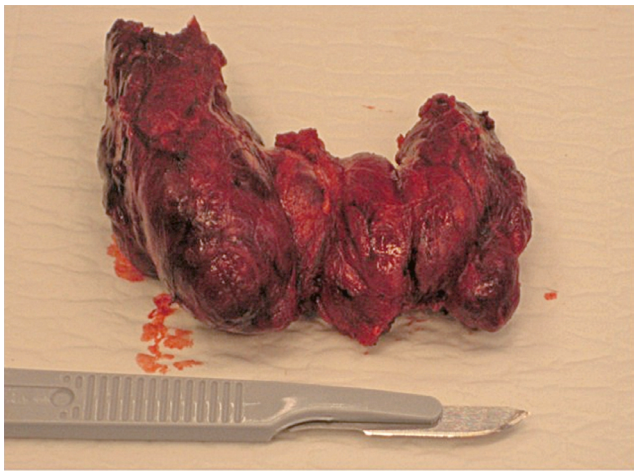


Figure 1. Surgically removed thyroid gland having an enlarged right lobe with suspected neoplastic growth. (Image from courtesy of Ilkka Heiskanen)

During embryogenesis the thyroid arises from the foramen caecum of the tongue as an endodermal structure and descends as a part of the thyroglossal duct, which usually atrophies, but remnants may be found in adults along this duct, such as in the ovaries (struma ovarii) (11-13).

The thyroid lobules are composed of follicles that contain colloid in their lumina. Colloid contains concentrated periodic acid-Schiff (PAS)-positive thyroglobulin. The polar-shaped thyroid hormone-producing follicular cells are arranged as a monolayer surrounding the colloid. Their nuclei are round, containing diffuse chromatin. Immunohistochemically, the follicular cells express thyroglobulin and thyroid transcription factor 1 (TTF-1), the building blocks for the thyroid hormones, low-molecular-weight keratin and vimentin.

The second type of hormone-producing cell, the C cells, accounting for only 0.1% of the thyroid´s mass, may be found as small clusters in the middle to upper third of the lateral lobes, usually in an intrafollicular position. The C cells produce calcitonin, a hormone

involved in calcium homeostasis, somatostatin and other peptides. Immunohistochemically, they accumulate calcitonin, TTF-1, somatostatin and neuroendocrine markers, such as chromogranin A and synaptophysin.

Solid cell nests are the third cell type of the normal thyroid gland and are believed to represent a remnant of the ultimobranchial body, from which the C cells are derived. The biological significance of these nests remains disputable (11-13).

The thyroid gland is a part of the hypothalamus-pituitary-thyroid (HPT) axis, with a feedback system activating from the hypothalamus, producing TRH (thyrotropin-releasing hormone), which activates the thyrotroph cells in the anterior lobe of the pituitary gland to release TSH (thyroid-stimulating hormone), which in turn activates the follicular cells of the thyroid gland to produce thyroid hormones called triiodothyroxine (T3) and tetraiodothyronine or thyroxine (T4). As a negative feedback mechanism, the hormones suppress the secretion of both TSH and TRH. The thyroid hormones have multiple actions in body metabolism, such as oxygen consumption, cardiac output and heat production.

5.2 Thyroid neoplasia

5.2.1 Incidence of thyroid nodules

General practitioners are often the first doctors who encounter a thyroid nodule presenting as a bulging of the neck. Thyroid nodules are common and their prevalence increases with age. Their prevalence is dependent largely on the method used for detecting the nodules. By palpation, an average worldwide prevalence of thyroid nodules has been estimated as 4–7% in iodine-sufficient regions (14-16), by US imaging up to 35–70% (14,16-18), and at autopsy from 50% to over 80% (14,16,19). With current diagnostic methods of clinically evident thyroid nodules, the risk of malignancy in all nodules is between 5% and 15%, whereas 15–20% are estimated to be benign adenomas and the rest adenomatous nodules (20-24). Incidentalomas, e.g. asymptomatic nonpalpable occult thyroid nodules, are becoming increasingly common, due to imaging of the neck for other reasons, such as computed tomography (CT) or angiography of the carotid arteries. The risk of malignancy in incidentalomas is 5–10% (14,25). However, not all incidentalomas lead to clinical disease, which is supported by the large quantity of occult cancers found in autopsy studies (14,16,19).

In comparing solitary nodule and multiple nodules, the prevalence of thyroid cancer is reported to be identical, when the nodules examined are larger than 10 mm in diameter. Nonetheless, cancer may appear in smaller nodules as well (22). Others have also reported a similarly identical cancer prevalence in multinodular goitre (MNG) and in solitary nodules, but irrespective of the size of the nodule (26). Contrasting results have been presented, with both higher and lower numbers of malignancies encountered in cases of solitary nodule

versus multinodular disease (18,27). An elevated risk of malignancy in relation or incidental to MNG has been shown. In a review, Nixon and Simo (18) estimated the incidence of MNG to be approximately 10% in the population when screened by US. The risk of occult cancer was estimated to be up to 10–35% in these occult MNGs. On the other hand, in a large meta-analysis the risk of thyroid cancer was less frequent in MNG than in a single nodule, in which the prevalence of thyroid cancer was estimated as 5% (27). Similar findings have been reported by other authors as well (26). Current practice considers goitre to be a benign disease with no need for routine follow-up.

Worldwide, thyroid cancer is the 18th most common cancer type among both genders, 9th in women and 21st in men, but it is the most common endocrine cancer (28). In Finland, thyroid cancer is the 13th most common cancer in women and 20th in men, accounting for 2.2% of all cancers in women and 0.7% in men (29). In the United States, these proportions are approximately 5% and 2% (30).

Currently the population of Finland is 5.4 million and the yearly number of new thyroid cancer cases is 350–400 (31). In Sweden with 9.5 million residents, the number of new thyroid cancer cases is approximately 430 per year (32) and in Norway with 5.0 million residents approximately 80 per year (33). Such variance in the incidence rates among the Nordic countries is surprising, without any clear explanation. Differences between diagnostic practice and diagnostic criteria may have an influence, as well as socioeconomic factors.

The National Cancer Institute Surveillance Epidemiology and End Results (SEER) programme has shown that thyroid cancer incidence rates in both genders have been increasing since 1980 in the United States. At the same time, cancer mortality has also increased, but at a much slower pace (34), or has even decreased (35). In Finland during the past 10 years, the incidence of thyroid cancer has increased by an average of 0.5% in men and 1.4% in women (29). Similar findings, especially of the increasing incidence of papillary subtypes in women, have been shown in almost all continents: Europe, the Americas, Asia and Oceania (35-37). Decreasing rates have been reported from Sweden, Norway and Spain (36), while in the Netherlands, the incidence seems to remain unchanged (38). An increase in the incidence of PTC cases and a decrease in FTC cases were seen in the Netherlands between 1989 and 2003 (38), whereas in Scotland the incidence of FTC increased between 1975 and 2000 (35). In Italy, there was no change in incidence between 1998 and 2009 (39). Interestingly, in Scotland the incidence in men is higher than in women (35).

In 1988, the WHO changed their histological classification of PTC according to nuclear features, which led to a shift of many carcinomas from FTC to FVPTC and to a decrease in the number of FTCs (40). FVPTC is predominantly comprised of a follicular architecture, with the majority of cells containing nuclei of pseudoinclusions and grooves (41). Molecular studies of FVPTC have shown a high prevalence of rat sarcoma (RAS) mutations, which are usually absent from the classical type of PTC, and a low prevalence of b-raf murine sarcoma viral oncogene homologue B1 (BRAF) mutations, which are often seen in PTC, thus suggesting a different origin of these two lesions (42).

In the United States, the most common thyroid carcinoma is PTC found as a microcarcinoma, mainly in patients older than 45 years (43). In Finland, microcarcinomas were detected in 36% of the cadavers of an autopsy study (19). An increasing incidence of microcarcinomas has been reported along with an increase in the occurrence of tumours over 4 cm in diameter, demonstrating a true increase in the incidence of thyroid cancer (36). On the other hand, it has been suggested that evolving diagnostic methods lead to discovery of smaller tumours than before, and thus detection of subclinical disease without a true increase in the occurrence of thyroid cancer (44). The explanation for the true increase in the incidence of thyroid cancer, especially PTC, may be the supplementation of iodine in food products, which is linked to autoimmune thyroiditis and again to PTC (45). Additionally, an increase in the use of bioaccumulating polybrominated diphenyl ethers (PBDEs), which are used as fire retardant coating matter in textiles and furniture, alters thyroid hormone homeostasis and causes thyroid dysfunction and tumorigenesis (46). In Finland, PBDEs are not manufactured, but the PBDE content of imported products is unknown (World Wildlife Fund, WWF). Increased exposure to external radiation, either therapeutic or accidental radiation, is linked to thyroid cancer (4). PTC especially is linked to ionizing radiation more often than other carcinoma subtypes and females are known to be more susceptible to it (5). A plausible explanation for the increasing incidence is the fact that the population is older than ever before in history, thus resulting in increased time to develop cancer during the lengthened human lifespan.

5.2.2 Classification of thyroid tumours

The classification of thyroid tumours according to the WHO into benign and malignant lesions is presented in Table 1.

Classification of Thyroid Tumours

Thyroid carcinomas	**Thyroid adenoma and related tumours**
Papillary carcinoma	Follicular adenoma
Follicular carcinoma	Hyalinizing trabecular tumour
Poorly differentiated carcinoma	**Other thyroid tumours**
Undifferentiated (anaplastic) carcinoma	Teratoma
Squamous-cell carcinoma	Primary lymphoma and plasmacytoma
Mucoepidermoid carcinoma	Ectopic thymoma
Sclerosing mucoepidermoid carcinoma with eosinophilia	Angiosarcoma
Mucinous carcinoma	Smooth muscle tumours
Medullary carcinoma	Peripheral nerve sheath tumours
Mixed medullary and follicular cell carcinoma	Paraganglioma
Spindle-cell tumour with thymus like differentiation	Solitary fibrous tumour
Carcinoma showing thymus like differentiation	Langerhans cell histiocytosis
	Secondary tumours

Table 1. WHO histological classification of thyroid tumours 2004 (2).

5.2.1.1 Benign tumours

Follicular thyroid adenoma

Epidemiology

FTA, a benign neoplasia derived from the follicular epithelium, is the most common neoplasia of the thyroid, with a 15–20% proportion of evident thyroid nodules (20,21). Radiation, iodine deficiency and history of nodular goitre are the major risk factors for follicular thyroid neoplasias (15). Some genetic alterations are shown in adenomas, such as mutations in RAS, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PI3KCA) and TSH receptor gene in toxic adenoma (15).

Clinical features

Adenomas are mainly nonfunctional. A small proportion shows autonomous hyperfunction (toxic adenoma), causing thyrotoxicosis. Tumours are usually found by palpation and may sometimes cause pressure effects, such as dysphagia and dyspnoea. Adenomas are five-fold more common in women than in men (15).

Histology

Microscopically, adenoma cells are regular arranged in a normo-, micro- or macrofollicular or trabecular growth pattern with uniform nuclear and cell morphology and rare mitotic cells. The oxyphilic (Hürtle cell, oncocytic) variant of FTA exhibits brightly eosinophilic granular cytoplasm and large open nuclei. Other variants of FTA are FTA with papillary hyperplasia, signet-ring cell FTA, mucinous FTA, lipoadenoma, clear cell FTA, toxic FTA and atypical FTA. Macroscopically, an adenoma is surrounded by a well-demarcated and intact capsule, has an average diameter of 3 cm and may resemble nodular goitre (11,12).

Diagnosis

At histology, invasion through the capsule or into a vessel is absent in benign neoplasias, distinguishing them from FTC. This diagnosis does not consider the possibility of a carcinoma in situ. Thus, tumours with a disturbed morphology, such as high cellularity and nuclear atypia, but no invasion, are referred to as atypical adenomas (15). Distinguishing FTA from adenomatoid (hyperplastic) nodules is arbitrary. Consistent criteria for their differential diagnosis are lacking. Adenomas are usually solitary nodules with a well-demarcated fibrous capsule. Adenomatous nodules are often multiple, lack a well-defined capsule and often show quite a similar morphology as the surrounding thyroid tissue (15).

Treatment

FTA is treated with lobectomy, including removal of the thyroid isthmus. Usually, hormone postoperative replacement therapy is not needed, since the other thyroid lobe is preserved. No follow-up is needed for these patients, since current knowledge is that these tumours seldom recur in the preserved lobe (11-13).

Hyalinizing trabecular tumour

This rare tumour group is of follicular cell origin, with a trabecular growth pattern and intratrabecular hyalinization, showing nuclear features related to PTC and rearranged during transfection (RET) rearrangement (RET/PTC). Microscopically, spindle cells are arranged in nests and psammoma bodies may be present. This tumour is usually considered as benign, but its relationship to PTC awakens suspicions, as well as some reported metastasized tumours (47,48).

5.2.2.2 Malignant tumours

The main subtypes of thyroid cancer include PTC and FTC (well-differentiated follicular epithelium-derived carcinomas [WDFTC]), medullary (MTC) and ATC in order of decreasing incidence. MTC is derived from C cells, while the other cancer types are derived from follicular epithelial cells. There is a new tumour entity called poorly differentiated follicular (PDFTC) or papillary thyroid carcinoma, with an incidence still largely unknown.

Papillary thyroid carcinoma

Accounting for up to 85% of thyroid cancers, PTC is the most common cancer subtype of the thyroid, with an often indolent course of disease with a 10-year survival of 95%. PTC occurs at ages 25–50 years, with female preponderance. The clinical presentation is often an asymptomatic thyroid nodule or cervical lymph node metastasis.

The worldwide incidence of PTC has risen for decades. The WHO introduced a new classification of thyroid neoplasia, in which FVPTC, with typical nuclear changes of PTC but with a follicular architecture, was classified as a subtype of PTC instead of a subtype of FTC, causing an increase in the number of PTCs. More sensitive and more commonly used imaging techniques have also led to detection of smaller lesions than before. Papillary microcarcinomas are incidentally found lesions measuring 1 cm or less in diameter, which are often nonencapsulated and are similarly indolent as larger PTCs.

Distinct genetic alterations unique for PTC have been found. The RET/PTC fusion protein is seen in 20–40% and neurotrophic tyrosine kinase, receptor, type 1 (NRTK1) mutation in 5–10%. BRAF gain-of-function mutation is seen in 30–50 % of PTCs, causing overactivation of the mitogen-activated protein (MAP) kinase pathway, leading to changes in cell growth, proliferation and differentiation.

Macroscopically, PTC may be encapsulated or infiltrate the surrounding tissues, showing fibrosis, calcifications and cysts. Histologically well-formed papillae and often psammoma bodies (concentrically calcified structures within the cores of papillae) are seen, as well as typical nuclear changes, which are hallmarks for cytological diagnosis of PTC, such as nuclear grooves and inclusions, and ground-glass nuclei (Orphan Annie eye). An invasion into the lymphatic vessels is typical for PTCs and lymph node metastases are seen in up to 25% of patients (41).

Follicular thyroid carcinoma

Epidemiology

FTC is the second most common thyroid cancer accounting for 10–15% of all thyroid cancers. Like PTC, it belongs to the group of WDTCs. FTC is three-fold more common in women, with occurrence peaking in the fifth decade, at slightly older age than in PTC patients. An average 5-year survival of FTC patients is 85–95%, depending on the aggressiveness of the tumour (49). In the developed countries the incidence of FTC has decreased, partly due to iodine supplementation in the diet, since iodine deficiency is an acknowledged risk factor for FTC. The WHO classification distinguishing FVPTC from FTC and categorizing them as belonging to the PTC group of carcinomas has decreased the incidence rates of FTC (49).

Symptoms

Typically, FTC presents as an asymptomatic slowly growing nodule in the neck with normal thyroid function. Local spread to the surrounding tissues may cause hoarseness, dysphagia and dyspnoea. Lymphatic spread is uncommon, while hematogenous spread is seen primarily to the bones, lungs or liver. The oxyphilic variant typically recurs in the neck and metastasizes more often than the conventional type (49).

Histology

FTCs are typically round or oval and are thickly encapsulated. Macroscopically, the tumours are light brown to pink in cut section, and areas of haemorrhage, necrosis and fibrosis may be evident. Microscopically, these carcinomas are usually quite uniform and hypercellular, showing some follicle formation with colloid, while a widely invasive subtype often presents with a solid or trabecular growth pattern. The tumour cells lack the nuclear features specific for PTC. Immunohistochemically, the cells are almost always thyroglobulin- and

TTF-1-positive indicating their well-differentiated status. The Hürtle cell variant of FTC is composed of oxyphilic cells with a typical appearance of abundant granular and eosinophilic cytoplasm filled with swollen mitochondria with scant or absent colloid. Clear cell change may be prominent in oncocytic neoplasias (40).

Classification

FTCs are divided into two categories: minimally invasive and widely invasive. The minimally invasive category includes tumours with focal capsular and/or vascular invasion. In widely invasive tumours, both capsular and vascular invasions are extensive. Capsular invasion protrudes through the entire thickness of the capsule. It should be distinguished from the cut made by FNAB. Vascular invasion is defined as evidence of tumour cells invading a vessel or cells inside a vessel surrounded by endothelial cells, in a vessel of the capsule or immediately around it. The tumour-node-metastasis (TNM) classification is used to determine the stage of the tumour (40) (Table 2,3).

TNM Classification	
T - Primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Tumour ≤ 2cm in greatest dimension, limited to the thyroid
T2	Tumour > 2cm, but ≤ 4cm in greatest dimension, limited to the thyroid
T3	Tumour > 4 cm in greatest dimension, limited to the thyroid or any tumour with minimal extrathyroidal extension, e.g. extension to sternohyoid muscle or perithyroid soft tissue
T4a	Tumour of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, oesophagus, or recurrent laryngeal nerve
T4b	Tumour invades prevertebral fascia or encases carotid artery or mediastinal nerves
All anaplastic tumours are considered T4	
T4a	*Intrathyroid - surgically resectable*
T4b	*Extrathyroid - surgically unresectable*
N - Regional lymph nodes	
Regional lymph nodes are the central compartment, lateral cervical and upper mediastinal nodes	
NX	Regional nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
N1a	Metastasis to level VI (pretracheal, paratracheal and prelaryngeal/Delphian) lymph nodes
N1b	Metastasis to unilateral, bilateral or contralateral cervical or superior mediastinal lymph nodes
M - Distant metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 2. The TNM classification of thyroid carcinomas (40).

Staging.			
Papillary or follicular carcinomas (under 45 years)			
Stage I	Any T	Any N	M0
Stage II	Any T	Any N	M1
Papillary or follicular carcinomas (45 years and older)			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1a	M0
	T2	N1a	M0
	T3	N1a	M0
Stage IV A	T4a	N0	M0
	T4a	N1a	M0
	T1	N1b	M0
	T2	N1b	M0
	T3	N1b	M0
	T4a	N1b	M0
Stage IV B	T4b	Any N	M0
Stage IV C	Any T	Any N	M1

Table 3. Staging of FTC and PTC according to the Union of International Cancer Control (UICC) TNM Classification (40).

Differential diagnostics

The group of follicular thyroid neoplasias includes both benign FTA and malignant FTC. Using current diagnostic methods distinguishing between these lesions is challenging. In cytology, they are both classified as follicular neoplasia, because no sufficient traits or markers are available for differentiation. Non-neoplastic lesions, such as adenomatoid nodules, may be mistaken for FTC if only cytological features are assessed. The diagnosis of FTC is made at histology by demonstration of a distinct capsulo- or vasculoinvasion. Occasionally, even morphological examination often misses a definite diagnosis. For example, according to this criterion, remaining in situ carcinomas cannot be found, with the only option being to wait for them to grow and eventually invade the surroundings (11,12).

Used alongside of routine examination, immunohistochemistry currently provides only mind bomb E3 ubiquitin protein ligase 1 (MIB-1/Ki-67) to aid in the differentiation between FTA and FTC (50). Numerous other markers have been studied, such as galectin-3, thyroid peroxidase (51), dipeptidyl peptidase 4 (DPP IV) (52), HBME-1 and cytokeratin-19 (CK-19) (53), to differentiate between FTA and FTC. None have shown enough diagnostic power to be recommended for clinical use (9,10).

Recently, molecular testing was shown to improve diagnostic sensitivity as well as cost-effectiveness, since it aids in preventing unnecessary diagnostic lobectomies (54). Molecular markers such as BRAF, RAS, RET/PTC or PAX8-PPARγ are recommended for testing in lesions with indetermined cytology. Currently, no molecular marker can predict a benign disease (55,56). BRAF, RET/PTC and RAS mutations are seen in 70% of PTCs, PAX8-PPARγ in 35–80% of FTCs and RAS mutation in 40–50% of FTCs. RAS mutation is also seen in 20–40% of FTAs and 10–20% of FVPTCs (57,58). These markers can predict thyroid malignancy with up to 100% certainty (positive predictive value), except for the RAS and PAX8-PPARγ mutations, which were also evidenced in FTAs. Such adenomas are believed to represent precursors for FTCs or in fact be in situ carcinomas or misdiagnosed FTCs, since these mutations are acknowledged as potent oncogenes and capable of promoting malignant transformation (57-60). The PAX8-PPARγ rearrangement is associated with angioinvasion in FTC. Accordingly, if a hyperplastic nodule harbours a RAS mutation, it should be considered a true neoplasia (57,58) (Table 4).

Papillary carcinoma	**Prevalence (%)**
BRAF	45
RET/PTC	20
RAS	10
TRK	<5
Follicular carcinoma	
RAS	45
PAX8-PPARγ	35
PI3KCA	<10
PTEN	<10
Poorly differentiated carcinoma	
RAS	35
b-Catenin	20
TP53	20
BRAF	20
AKT1	15
Anaplastic carcinoma	
TP53	70
b-Catenin	60
RAS	50
BRAF	20
PI3KCA	20
PTEN	>10

Table 4. Prevalence of molecular mutations in thyroid carcinomas. Modified from (61).

Molecular testing of multiple alterations may be used to classify undetermined FNAB cases as low- and high-risk categories, thus increasing diagnostic accuracy (61). However, the diagnostic utility of molecular testing faces major obstacles, the most important being that morphology is lost during processing. Indeed, currently the best diagnostic accuracy seems to be obtained with traditional morphology (10). Moreover the number of cells needed is considerably high and unlikely to be obtained from every FNAB sample (62), not to mention that molecular testing suffers from high costs and is time-consuming.

Treatment

In the case of a suspected follicular neoplasia at cytology, a lobectomy of the thyroid gland is performed and diagnosis of FTC is made at histology. The treatment for FTC includes total thyroidectomy followed by I131 ablation to eradicate any tumour cells remaining, as well as normal thyroid cells, after surgery. The treatment is continued with high-dose thyroxin treatment to suppress TSH levels to a minimum, due to growth factor abilities of TSH on remaining tumour cells in the body. Additional surgery and radioiodine, radiotherapy and chemotherapy may be administered in metastatic diseases.

Prognosis

The prognosis for patients with the minimally invasive subtype of FTC is excellent; 5-year survival is over 90%. The prognosis for widely invasive FTC is dependent on the extent of the disease and the presence of distant metastases, decreasing the 5-year survival to 50% (11,49).

Poorly differentiated thyroid carcinoma

This cancer subtype, also known as insular, solid or trabecular carcinoma, was first introduced in 2004 by the WHO, distinguishing it from well-differentiated and undifferentiated carcinomas of the thyroid as an intermediate between them both histologically and behaviourally. PDTC may emerge from either PTC or FTC, and accordingly the entire tumour should be diagnosed as poorly differentiated, even if only a small proportion of it exhibits poor differentiation traits, because some of these tumours behave like PDTC (63,64). The estimated incidence is 4–7% of all thyroid carcinomas. PDTC often presents with metastases to the lungs, liver or bone and has a 5-year survival of approximately 50% (65).

PDTC is a tumour showing a solid, trabecular or insular growth pattern, with convoluted nuclei, high mitotic activity or tumour necrosis (66). The tumours show high cellularity and scant colloid. The nuclei are bland, containing fine chromatin and small nucleoli, but focal nuclear pleomorphism may be present. Necrosis and mitoses are common, while atypia are

mild compared with that of ATCs. A limited amount of follicular cell differentiation, e.g. thyroglobulin and TTF-1 immunoreactivity, is present.

Anaplastic thyroid carcinoma

ATC, e.g. undifferentiated carcinomas, develop from the follicular epithelium and account for less than 5% of thyroid carcinomas. Genetic alterations, such as loss-of-function mutation in the TP53 gene, are commonly found. The 5-year survival is poor, ranging from 0% to 14% (67). The clinical course is aggressive, with a rapidly growing tumour in the neck, which at the time of diagnosis has often infiltrated to the surrounding tissues or metastasized to the lungs, bone or brain. ATC is more commonly seen in the elderly and has an even gender distribution. Histologically, the tumours appear with extensive mitotic activity, necrosis and high cellularity. Immunohistochemically, markers of differentiation, such as thyroglobulin, are expressed focally if at all.

Medullary carcinoma

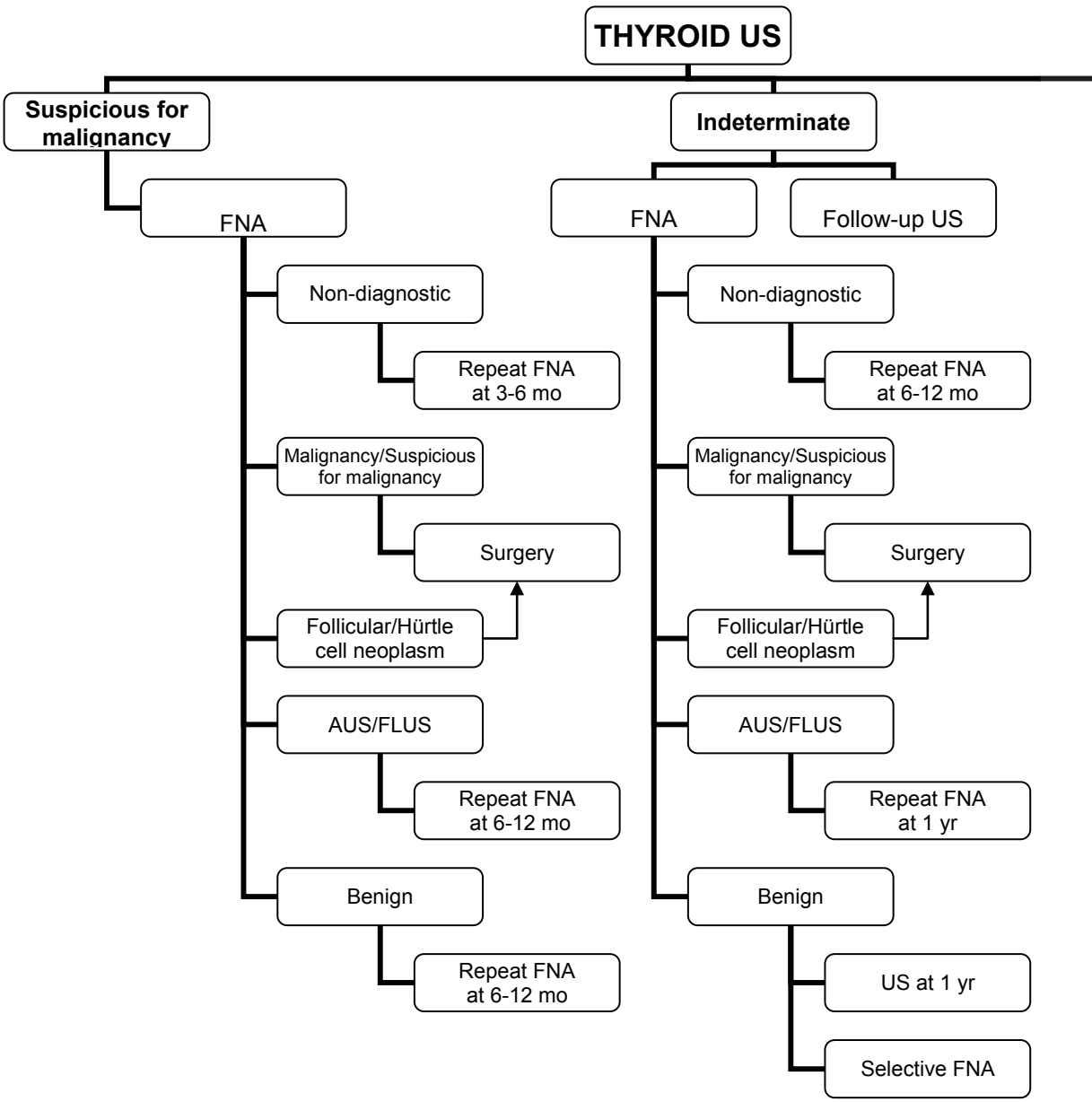
MTC, which constitutes about 5% of thyroid carcinomas, is derived from C cells and is considered a neuroendocrine neoplasia. It secretes calcitonin, and elevated concentrations may be measured from the blood, while the tumours are calcitonin-positive at immunohistochemistry. MTC may be either sporadic or familial, the latter being part of the multiple endocrine neoplasia 2 (MEN2) syndrome. An average 5-year survival is approximately 80% (68).

5.2.3 Diagnosis and treatment of thyroid cancer

The current practice of managing a thyroid nodule, either incidental or symptomatic, is to perform high-resolution US including FNAB, and to evaluate the levels of TSH and T4. A neoplastic growth is most often presented in conjunction with euthyroidal hormone status. Hypo- or hyperthyreosis causes cell abnormality and nodular formation, which are often reversible, once the patient is treated. An elevated TSH level has been associated with an increased risk of malignancy (69), and the American Thyroid Association (ATA) recommends evaluation of such nodules (70). On the other hand, contrasting results have been shown (71).

To characterize a nodule, selective US targeting is used for improving the adequacy of the sample and increasing the accuracy by inserting the needle into a solid portion of the nodule. The nodules are evaluated for size, internal content (cystic, solid), shape, appearance of the nodule margin, presentation of a halo surrounding the tumour, echogeneity, calcifications, extracapsular invasion and tumour vascularity with a doppler device.

Cervical lymphadenopathy is also evaluated. Suggestive of malignancy is a hypoechoic solid nodule representing an irregular/spiculated margin without a halo surrounding it, showing intratumoral vascularity and microcalcifications. A taller-than-wider shape is also a malignant feature, since cancer tends to grow in a centrifugal way, while benign nodules grow parallel to the tissue surface. A diameter over a cut-point of 1 cm of the thyroid nodule size may be an indication for biopsy. Nodules below this diameter should be assessed in cases of a high-risk medical history (rapid growth and fixation of the nodule to the surrounding



tissues, hoarseness, elevated TSH level, lymphadenopathy, head and neck irradiation, exposure to ionizing radiation, positive family history or a thyroid cancer syndrome) or suspicious findings on the US examination previously mentioned or multifocality, bilaterality or increase in size during follow-up (21,55,70,72). Smaller nodules should only be followed up, because biopsy of such small nodules often indicates a high false-positive rate (72,73), (Figure 2).

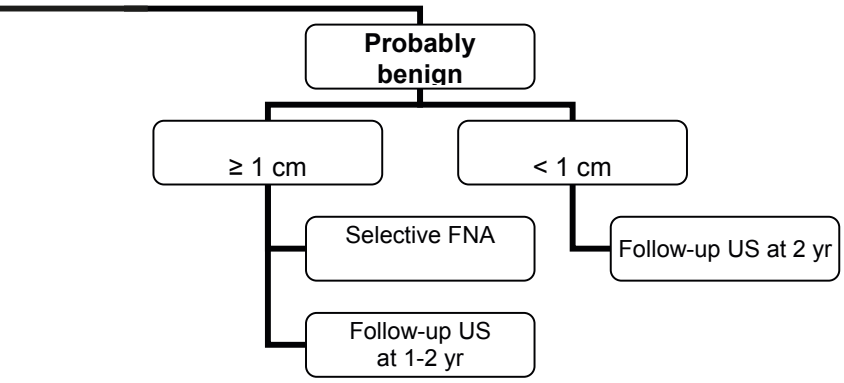


Figure 2. Flow chart introducing a management recommendation in thyroid ultrasonography and fine-needle biopsy. US = ultrasonography, AUS = atypia of undetermined significance, FLUS = follicular lesion of undetermined significance. Modified from (21,70,72).

Recent studies have shown that the size requirement for FNAB indication should be reassessed. Some studies showed that even nodules at least 4 mm in size should be biopsied irrespective of their clinical or US characteristics, because a considerable number of such microcarcinomas were shown with the BRAFV600E mutation, a molecular marker linked to aggressiveness in PTC, or aggressive traits such as nodal metastases (54,74-76).

FNAB is the method of choice in diagnosing thyroid nodules. Large-needle or core-needle biopses are to be avoided because the thyroid gland is a highly vascularized organ with the risk of bleeding and local pain, and no extra benefit is gained compared with use of FNAB. On the other hand, some studies showed that after one FNAB with inconclusive results (nondiagnostic or atypia of undetermined significance (AUS) or follicular lesion of undetermined significance (FLUS), core-needle biopsy would be more useful than repeated FNAB for reducing the frequency of inconclusive diagnostic results (73,77).

FNAB is useful for diagnosing PTC. On the other hand, follicular neoplasias present a challenge, since FTC and FTA cannot be differentiated at cytology. Because of limited sensitivity and specificity, FNAB suffers from a considerable number of false-negative and false-positive results leading to unnecessary surgery (78). The majority of FNABs performed show a benign tumour (60–80%), 10-20% a follicular lesion, 10–15% are nondiagnostic, up to 10% are suspected malignancies and up to 10% are malignant at cytology (54,73).

Due to diversity in terminology and morphologic criteria in FNAB assessment, a new classification system for reporting thyroid cytology was introduced in 2010 (The Bethesda System for Reporting Thyroid Cytopathology, (79) (Table 5). This system increases the cytologic-histologic correlation and offers recommendations for clinical management of thyroid nodules.

A follicular neoplasia is counted in two of Bethesda´s categories. The preferred category is follicular neoplasm or suspected follicular neoplasia, which holds a 15–30% risk of malignancy, mainly due to FTC or FVPTC. The majority of cases in this category are FTAs or adenomatoid nodules of MNG. The purpose of this category is to triage an FTC to surgery. Cytologically, the architecture of the cells is atypical, the cells are arranged in a crowded microfollicular or trabecular manner and the nuclear features of PTC in their slightly enlarged nuclei are absent. A low mitotic number is normally seen. If the majority of the cells of the biopsy show Hürtle cell-type features, about 15–45% of such nodules are evidenced malignant (79).

Additionally, follicular neoplasias may be classified in the category of AUS or FLUS, which is used when the sample cannot be classified in any distinct category. Although this category may cause anxiety to patients and clinicians before better neoplasia-specific markers are found, it is of great value as a follow-up and repeated biopsy may reveal the true nature of the lesion. Thus, both unnecessary surgery, due to false-positive cases, as well as true neoplasias presenting as false-negative cases, can be diminished, using the AUS category (79).

Once a patient with a thyroid nodule is directed to surgery, the management is individualized with disease staging and risk factors are taken into account (Table 2). In the case of a

The Bethesda System	Risk of malignancy (%)	Recommended procedure
Nondiagnostic or Unsatisfactory Cyst fluid only Virtually acellular specimen Other (e.g. obscuring blood, clotting artefact)	**1–4**	**New biopsy**
Benign Consistent with a benign follicular nodule (e.g. adenomatoid nodule, colloid nodule) Consistent with lymphocytic (Hashimoto) thyroiditis in the proper clinical context Consistent with granulomatous (subacute) thyroiditis Other	**0–3**	**Clinical follow-up**
Atypia of Undetermined Significance or Follicular Lesion of Undetermined Significance	**5–15**	**New biopsy**
Follicular Neoplasm or Suspicious for a Follicular Neoplasm Specify if Hürtle cell (oncocytic) type	**15–30**	**Surgical lobectomy** No frozen section recommended
Suspicious for Malignancy Suspicious for papillary carcinoma Suspicious for medullary carcinoma Suspicious for metastatic carcinoma Suspicious for lymphoma Other	**60–75**	**Surgical lobectomy/ thyroidectomy** Frozen sections may help during the surgery
Malignant Papillary thyroid carcinoma Poorly differentiated carcinoma Medullary thyroid carcinoma Undifferentiated (anaplastic) carcinoma Squamous cell carcinoma Carcinoma with mixed features Metastatic carcinoma Non-Hodgkin lymphoma Other	**97–99**	**Thyroidectomy**

Table 5. The Bethesda System for Reporting Thyroid Cytopathology (79).

suspected follicular neoplasia, a primary lobectomy is performed (80). If a diagnosis of FTC at histology is made, a complementary thyroidectomy will be performed. If a single occult papillary microcarcinoma without lymph node metastasis is incidentally found, a lobectomy may be considered sufficient treatment (80). A primary total thyroidectomy will be performed if malignancy is evidenced in FNAB; in a suspected malignancy a frozen section will be performed during surgery for verification of a malignancy (21) (Table 5).

The extent of surgery is dependent on the spread of disease to the surrounding tissues. The completeness of the surgical resection is important, because it defines the outcome of the disease (70). Due to growing morbidity in PTC, a lateral lymph node dissection of the neck is not commonly recommended if US imaging of lymph nodes of the neck is found negative. Most experts, on the other hand, routinely advocate a central dissection of at least the T3 or T4 primary lesions, because central lymph node metastases in the thyroid bed are common, their detection with US is uncertain and dissection improves staging accuracy and decision for radioiodine administration (81). Previously, it was believed that lymph node metastasis had no effect on prognosis, but this argument has been overruled in support of a more concise dissection of lymph nodes (81).

Following thyroidectomy, in approximately 4 weeks radioactive iodine ablation (I131) of 1110–3700 MBq is performed for patients with PTC or FTC to destroy any remaining thyroid tissue and possible micrometastases, because normal thyroid cells and well-differentiated carcinoma cells also concentrate radioactive iodine (82). In Finland, I131 is administered for all WDFTC patients with a tumour above 1 cm in diameter or if disease is multifocal or metastases are present. I131 is not given to patients with PDTC or ATC, because these cancers seldom concentrate iodine due to disease dedifferentiation. In PDTC, ablation may be considered if the disease progresses. Ablation is given under TSH stimulation by thyroxin deprivation or administration of rhTSH. Ablation is repeated if remnant thyroid tissue is evident 6–8 months after the first dose during routine follow-up, as discovered with gamma and US imaging and serum thyroglobulin measurement. The thyroxin dose is kept high to suppress the trophic effect of TSH. Metastases and neck recurrences are treated, if possible, with secondary surgery. Other treatments for less differentiated and metastasized diseases include external radiation and chemotherapy which are often given both pre- and postoperatively. New treatments such as tyrosine kinase inhibitors have been studied (83).

Thyroid carcinoma patients are monitored by yearly follow-up, including clinical examination, measurements of non-TSH-stimulated thyroglobulin, TSH and free thyroxin or T3 and US imaging of the neck (every 2 years) to ensure a disease-free state. If these examinations prove positive, gamma imaging with an 185–MBq dose and TSH-stimulated thyroglobulin measurement (including measurement of antithyroglobulin antibodies) will be pursued (21). Other imaging methods are also used if necessary, such as positron emission tomography (PET) using fluoro-18-deoxyglucose or CT. Follow-up should be lifelong, since recurrences may occur many years after initial therapy.

5.3 Tumorigenesis and genetics of follicular thyroid tumours

Genetic cancer susceptibility is more common among endocrine tumours than any other human neoplasia (2). Six percent of familial nonmedullary thyroid cancers (FNMTCs) are believed to be familial follicular thyroid cancers (FFTCs), although no diagnostic criteria have been established. FFTC may be associated with syndromes such as Cowden or Werner

syndromes or the Carney complex. Mutations in the phosphatase and tensin homologue (PTEN) tumoursuppressor gene cause the Cowden syndrome, with susceptibility to breast, endometrial, thyroid, kidney and colorectal cancers, including dermatologic, gastrointestinal and neurologic features. Up to 10% of patients with Cowden syndrome develop a thyroid cancer, usually an FTC. Werner syndrome, also known as adult progeria, caused by a mutation in the Werner syndrome, RecQ helicaselike (WRN) gene, causing premature aging in patients, is associated with an increased risk of cancer, including FTC. The Carney complex, caused by mutations in protein kinase, cyclic adenosine monophosphate (cAMP)-dependent, regulatory, type 1, alpha (PRKAR1A) gene or chromosome 2p16, includes neoplasias in the heart, skin, endocrine organs such as the thyroid, and pigmentations of the skin and endocrinopathy.

Tumorigenesis of FTC is a much-debated topic. Suggestions of de novo mutations in thyroid cells, forming a carcinoma, are common. The most popular theory of classical multistep carcinogenesis suggests a precursor formation from an FTA, which acquires genetic alterations to give the tumour a growth-promoting and an invasive phenotype (Figure 3). However, these concepts are challenged, because the proliferation rate of adult thyrocytes is low, thus limiting the time for accumulation of genetic alterations. In contrast, during goitrogenesis an immense increase in thyroid cell number occurs, which leads to a burst of mutational events and a failure of mutation repair systems during the higher replication pace. However, the spontaneous mutation rate in the thyroid is much higher than in other organs of mice at least (55). Another point is the wide heterogeneity of thyroid carcinomas, which are unlikely to be caused by only one mutation, which may once again be argued because mutations may occur at any site of the pathway, leading to similar end-events. However, when the DNA copy number of follicular thyroid neoplasias was characterized, FTAs showed mainly gains in genetic material, whereas FTCs showed deletions, but identical alterations were also seen, agreeing with the classical multistep concept (84,85). Previous studies showed that goitre and benign adenomas were strong risk factors for thyroid cancer (18,22,86). A carcinoma arising within a goitrous thyroid gland is smaller and develops at an older age then in patients whose solitary carcinoma nodules arise de novo (87). Genetic studies propose a pattern of cumulative alterations in tumour progression. Furthermore, it has also been suggested that a minimally invasive FTC is a precursor to widely invasive FTC (88,89).

Follicular cell-derived neoplasias to have genetic alterations in at least two distinct intracellular signaling pathways, leading to uncontrolled growth: the MAP kinase pathway and the phosphatidylinositol-3-kinase-protein kinase B (PI3K-AKT) pathway. Up to 30–50% of FTCs, especially less-differentiated FTCs, harbour mutations of the latter pathway, such as gain-of-function point mutations in the RAS and PI3K genes and loss-of-function mutation in the PTEN, β-catenin and tumour protein 53 (TP53) genes. A progressive increase in the rate of RAS and PI3KCA mutations may be the common pathogenesis of FTAs transforming to FTCs and further to ATCs. A RAS mutation, which is the most common mutation in FTCs, is seen in 40–50% of conventional FTCs and in 20–40% of conventional FTAs (23,58).

A fusion gene of paired box gene 8-peroxisome proliferator-activated receptor γ (PAX8-PPARγ), which causes loss of growth inhibitory control in the PPARγ gene, is seen in 30–50% of FTCs but also in 10% of FTAs (12). However, in FTCs it is associated with vasculoinvasion and metastatic disease (90-92). The RAS and PPARγ-PAX8 mutations do not overlap, which suggests two distinct pathways of tumorigenesis. Lately, new factors have been found affecting the pathogenesis of FTC, called microRNAs (miRNAs), which are small noncoding segments of ribonucleic acid (RNA) important in negatively regulating gene transcription (93).

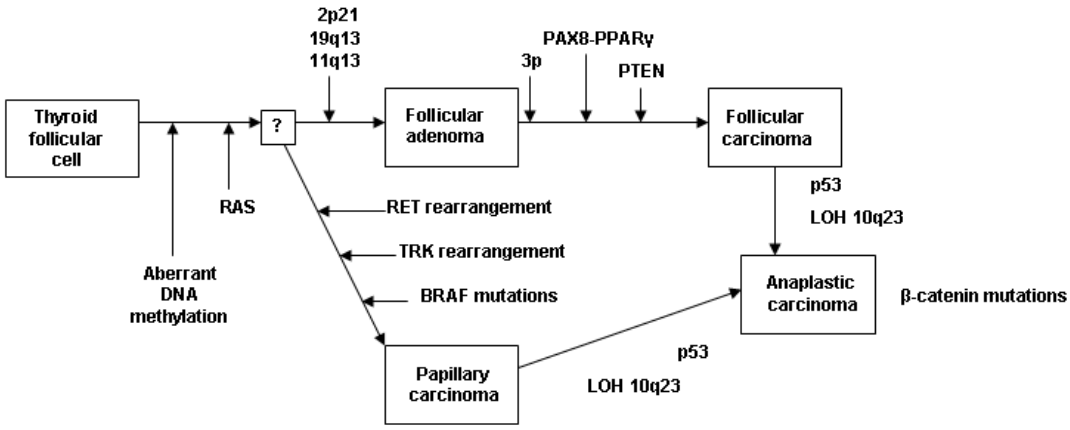


Figure 3. Tumorigenesis of thyroid neoplasias. Modified from (11,59,94-96).

Neoplasias are composed of genetically heterogeneous cells, each with different phenotypic characteristics and different proliferative potentials. According to a new hypothesis, a small subset of cells called cancer stem cells (CSCs) plays a major role in carcinogenesis (85). These undifferentiated and pluripotent cells originate from either normal stem cells through genetic alterations or from progenitor cells or adult normal cells via a dedifferentiation process called the epithelial to mesenchymal transition (EMT) (97). These cells may rest as dormant cells, self-renew or produce a progenitor cell that differentiates into either a well-differentiated or an undifferentiated cancer cell and eventually multiplies and forms the bulk of the tumour (98). Dormant and quiescent CSCs without active growth signalling are inert to traditional treatments, because these treatments are directed against rapidly dividing cells (99).

CSCs have been isolated from PTC, FTC and ATC (100). In normal thyroid tissue only 0.1% of cells are evidenced as stem cells, while 1–2% are in goitrous tissue (99). CSCs with different potentials for proliferating may explain the thyroid tumorigenesis in different carcinoma subtypes (98,101). Fetal CSCs without any markers of differentiation are believed to give rise to ATC, while thyroblasts with both fetal and differentiation markers give rise to PTC and prothyrocytes with differentiation markers but without fetal markers give rise to FTC (101). Immatureness of the CSC population, may be linked with the degree of cancer malignancy; in ATC the stronger the expression of cluster of differentiation 133 (CD133), a stem cell marker, the stronger is the ability of carcinoma to initiate tumours compared with low expression (101).

5.4 Tumour markers of follicular thyroid neoplasias

Hanahan and Weinberg (102) listed nine hallmarks of cancer to understand the complex process of human neoplasia. The most constitutive hallmark is the ability to constantly proliferate. Proliferation is activated by growth factors in an autocrine manner or by disrupting self-attenuating signalling, which is carefully controlled in normal cells. The remaining hallmarks include evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, inflammation, reprogramming of energy metabolism and evading immune destruction. Carcinogenesis is believed to proceed as a sequential accumulation of alterations in genes such as oncogenes, growth suppressor genes, growth factors and growth factor receptor genes involved in these processes, leading to a colony of genetically modified tumour cells with a growth advantage over normal cells. Several immunohistochemical protein markers indicating these hallmarks have been studied in the field of thyroid neoplasia (Figure 4, Table 6).

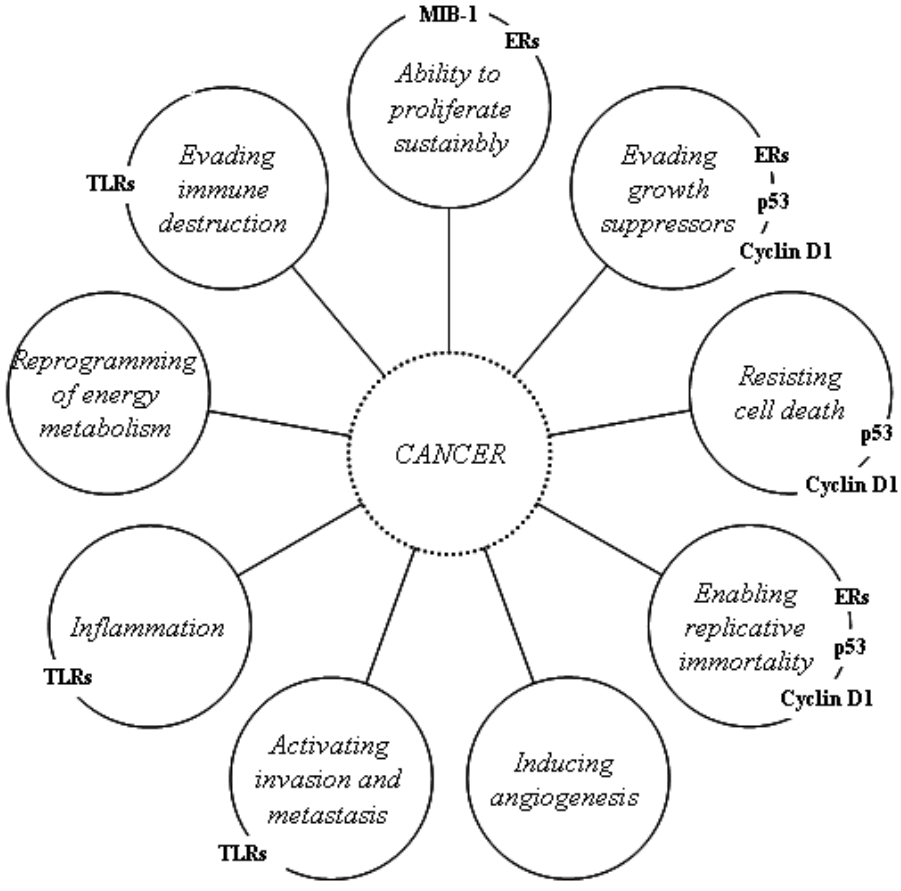


Figure 4. Hallmarks of cancer by Hanahan and Weinberg (102). Markers studied are next to the hallmarks in question.

Marker	Role	FTA
MIB-1	Detection of highly proliferative tumours	<5% positive cells
TPO	Confirming thyroid origin of tissue	Positive
TTF-1	Confirming thyroid origin of tissue	Positive
RAS mutation	Dedifferentiation, aggressive behaviour	Positive
PAX8-PPARγ	Carcinogenesis, vascular invasion	Atypical FTAs positive
Cyclin D1	Carcinogenesis, metastasis	Low positivity
ERα	Tumorigenesis, aggressive behaviour	Low positivity
ERβ	Tumorigenesis, aggressive behaviour	Positive
p27	Dedifferentiation, poor prognosis	Positive
p53	Dedifferentiation, aggressive behaviour	Negative
Telomerase	Tumour development, aggressive behaviour	Negative
Galectin-3	Tumour progression, metastasis	Negative
E-cadherin	Invasiveness, poor prognosis	Positive
β-catenin	Dedifferentiation, aggressive behaviour, poor prognosis	Positive
Cytokeratin-19	Identification of PTC	Focal positivity
HBME-1	Carcinogenesis, metastasis	Low positivity
TGF	Tumorigenesis	Positive
IMP-3	Tumorigenesis, metastasis, poor prognosis	Negative
COX-2	Tumorigenesis	Low positivity
VEGF	Tumorigenesis, metastasis, poor prognosis	Low positivity
EGF	Tumorigenesis, aggressive behaviour	Low positivity
MMP	Invasion, metastasis	Low positivity

Table 6. Some markers studied in follicular thyroid neoplasia

5.4.1 Ability to proliferate

The growth activity of thyroid tumour cells is inversely correlated with the differentiation level of carcinomas (135). The proliferation indexes (PIs) of MIB-1 antibody (Ki-67 antigen) are 1–5%, 5–10%, 10–30% and >30% in the spot areas of WDTC, PDTC and ATC, respectively. MIB-1 is an essential tool in clinical pathology, indicating the proliferative ability of the tumour. It has prognostic and predictive value and is more commonly used in the diagnostics of PDTCs and ATCs (103-105,113,136).

TPO is commonly used in clinical diagnostics to confirm tissue, as it is a thyroid-specific enzyme involved in the synthesis of thyroid hormone and expressed solely in thyroid tissue. Its expression is focally lost in PDTCs, but it is mainly present in well-differentiated carcinomas, adenomas and normal thyroid tissue. Thus, the absence of TPO indicates loss of

WDFTC	PDFTC	Ref
5-10% positive cells	10-30% positive cells	(103-105)
Positive	Focal positivity	(106,107)
Positive	Focal positivity	(106,108,109)
Positive	Often positive	(23,58,96,110,111)
Positive	Rarely positive	(90,111,112)
Intermediate positivity	High positivity	(113)
Intermediate positivity	High positivity	(114-117)
Intermediate positivity	Low positivity	(114-118)
Intermediate positivity	Negative	(103,105,113,119)
Negative	20-30% positive cells	(95,96,103,112,120,121)
Intermediate positivity	High positivity	(112,122)
Intermediate positivity	High positivity	(53,106,119,123)
Intermediate positivity	Negative	(124,125)
Intermediate positivity	Negative	(106,112)
Intermediate positivity	Low positivity	(106,123,126)
Positive	Positive	(53,106,127)
Intermediate positivity	Low positivity	(128)
Positive	Positive	(129,130)
Intermediate positivity	High positivity	(106,131)
Intermediate positivity	Positive	(127,132)
Intermediate positivity	Positive	(96,132,133)
Intermediate positivity	Positive	(127,134)

differentiation of tumour cells. It has predictive value of the effectiveness of the treatment as radioiodine uptake is poor if the expression of TPO is low (106,107). Thyroid transcription factor -1 (TTF-1) is also a protein expressed in the thyroid. It is also found in the brain, lungs and MTC. It is commonly used in clinical practice in the same purpose as TPO. TTF-1 expression is similarily lost in PDTC and ATC (106,108,109).

Thyroglobulin detection is made both from serum and histologic sample. Serum level is a well-acknowledged marker predicting poor survival (137), whereas immunohistochemical staining is performed to confirm the origin of tissue similarly as TPO and TTF-1. Measurement of repeated serum thyroglobulin level predicts the long-term outcome of differentiated thyroid cancer patients (138).

5.4.2 Enabling replicative immortality

A genetic mutation in oncogenes generate uncontrolled growth signals and cause cancer cells to develop and proliferate. Several oncogenes have been studied in thyroid cancer. In follicular neoplasias, the main alterations are in RAS and PAX8 genes. On the contrary, BRAF and RET/PTC mutations are specific to PTC (58). Activating point mutations in RAS genes (H-RAS, K-RAS, N-RAS), encoding intracellular G-proteins, involved in signal transmitting in MAP and PI3K-AKT pathways, are absent from non-neoplastic thyroid lesions. They are mainly seen in follicular neoplasias and only rarely in PTC. Virtually all PTCs positive are follicular variants (23,58,110). RAS mutations are linked to iodine deficiency and are thought to represent an early event in thyroid tumorigenesis. Futhermore, RAS mutations are correlated with the traits of dedifferentiation traits, such as diminishing thyroglobulin expression and aggressive behaviour (96,111,112). Carcinomas with RAS mutation are shown to metastasize more often than carcinomas without RAS mutations (58).

Paired box gene 8 (PAX8) encodes a transcription factor involved in the regulation of cell cycle, DNA repair, metabolism, replication and cell polarity. Its protein expression is shown to be decreased or lost in FTC (90). PPARγ is a tumour suppressor gene which stimulates lipogenesis, promotes differentiation and inhibits cell proliferation. A fusion gene PAX8-PPARγ, arises from translocation t(2;3)(q13;p25) and leads to suppression of the function of PPARγ gene (90). It is seen in FTCs, FTAs and FVPTCs, but not in conventional PTCs. FTAs with PAX8-PPARγ translocation might be precursors for FTCs since this mutation was seen in a small group of FTAs, which evidently show atypical features. FTCs harbouring PAX8-PPARγ translocation are smaller in size, present at younger age and are more vasculoinvasive, whereas FTCs harbouring RAS mutations are larger and present at older age (111). Interestingly these two genetic alterations are rarely seen in Hürtle cell neoplasias, which are thought to represent a distinct type of thyroid neoplasia.

Nikiforova et al have suggested the use of these two distinct molecular markers in FNAB diagnostics: RAS as a neoplasia marker and PAX8-PPARγ as a malignancy marker (111), although opposing opinions have also been presented (60). The American Thyroid Association (ATA) recommends the use of molecular markers such as BRAF, RET/PTC, RAS, and PAX8-PPARγ, in evaluating the thyroid nodules with undetermined cytology (70).

Cell cycle related genes include promoters, such as Cyclin D and E and inhibitors, such as p27, p53 and retinoblastoma (RB) protein. The promoters are considered oncogenes and the inhibitors tumour suppressor genes. The most studied promoter of the cell cycle is Cyclin D1, encoded by human CCND1 gene, which is one of the most frequently amplified genes in human cancers. It is a key regulator of the G1/S transition through the cell cycle by inactivating RB protein. Cyclin D1 is increased just before the S phase in normal cells, while in neoplastic cells it is highly overexpessed. In thyroid, it is commonly mildly expressed in well-differentiated carcinomas, intermediately in poorly differentiated and highly expressed in metastatic PTC and ATC (65,113). It was expressed stronger in FTC than in FTA in one study (113), although the result could not be repeated (139).

Hormones and hormone receptors, especially estrogens, are a much-studied topic in thyroid cancer as thyroid diseases are often connected to female gender. Estrogen receptor subtypes α and β are involved in cancer cell proliferation and are thought to oppose one another´s action: α induces proliferation, whereas β suppresses it (140). According to previous studies ERβ is a tumour suppressor inhibiting cell´s dedifferentiation maintaining the cell in its primary phenotype. The prognostic association with ERβ has also reported in breast cancer, ovarian cancer, and lung cancer (141-143). Both α and β subtypes are expressed in normal as well as neoplastic thyroid tissue. The expression of α receptor has shown both higher and lower expression intensities in carcinoma tissue compared to non-neoplastic thyroid tissue (114,115,144). On the other hand, β receptor is expressed stronger in FTC than in FTA (114), although contradictory results have been published (115), as well as results with no difference (116,118).

5.4.3 Evading growth suppressors

Tumour suppressor genes suppress the action of oncogenes. A mutation in these recessive genes cause loss of the suppressive action through a phenomenon called loss of heterozygosity (LOH). LOH in tumour suppressor genes is more often seen in FTC than in PTC (96).

The cyclin dependent kinases (CDK), which facilitate the progression of the cell cycle, are activated by cyclins and inhibited by CDK inhibitors such as protein 27 (p27). P27 inhibits the cell cycle by activating RB protein, affecting the same phase in the cell cycle as Cyclin D1. P27 is shown to be downregulated in thyroid cancer, especially in PTC, and maybe helpful in differential diagnostics of follicular neoplasias. It has also shown prognostic value when comparing WDTC to PDTC (103,105,113,119).

5.4.4 Resisting cell death

The tumour-suppressor gene TP53 is defined as "the guardian of the genome" with a main function to arrest the cell cycle in G1 phase in the occurrence of DNA damage enabling the repair mechanisms to correct the damage. In the case of failed repair, the cell would proceed to apoptosis activated by protein 53 (p53). Conversely, loss-of-function mutation of the TP53 gene leads to resistance of cell death inducing genomic instability, prolonged cell life and cancer progression. Overexpression of mutated p53 is seen abundantly in ATC and in lower amounts in PDTCs, while it is nearly absent in WDTCs thus supporting the classical theory of dedifferentiation process from a WDTC to PDTC and eventually to ATC (135). Overexpression of p53 is a marker for those tumours with an ability to behave aggressively as well as an independent prognostic factor for overall survival of patients with thyroid carcinoma (65,95,96,103,120,121).

Telomeres are thought to be the molecular clock for ageing of human cells. These end parts of the chromosomes are shortened during every cell cycle and eventually run out, which activates cell apoptosis. Telomerase enzyme is responsible for maintaining the length of telomeres in the ends of chromosomes. Activity of the telomerase enzyme is shown in a majority of all types of human cancer (80-90%). Normal thyroid tissue is shown to lack telomerase activity, whereas in malignant cells it is upregulated, especially in PDTC. It may be a marker of aggressiveness and involved in the progression from WDTC to PDTC (112,122).

MicroRNAs (miRNAs) are small non-coding RNA molecules that can modulate gene expression by binding to the target messenger RNA, which can result in the decreased expression of the target protein. Recent evidence has shown that these molecules are involved in initiation and progression of several types of cancer. In thyroid cancer some miRNAs (miR-7 and miR-200b) are shown to be exclusive for FTC (145).

5.4.5 Activating invasion and metastasis

Altered adhesion molecules correlate with tumour progression, especially invasiveness and metastatic potential. Expression of such molecules is lost in widely invasive and metastatic thyroid carcinomas. Galectin-3, a member of the adhesion molecules called lectins, plays an important role in cell-cell/matrix interactions, adhesion, migration and repairing of damaged cells. It is expressed in WDTCs, especially in PTC and less intensely in FTC, but it is absent or weakly expressed in adenomas and non-neoplastic thyroid tissue. PDTC and ATC strongly express galectin-3. It may help in the differentiation between minimally invasive FTC and FTA (53,106,119,123).

The expression of e-cadherin, a transmembrane glycoprotein adhesion molecule essential for maintaining intercellular junctions, is shown to be lower in widely invasive and metastatic compared to minimally invasive FTCs. It is correlated with poor prognosis of thyroid cancer (124,125). Membranous expression of β-catenin, an adhesion molecule connected to e-cadherin, is lost in PDTC and ATC indicating loss of differentiation. It is connected to poor prognosis, as well (106,112).

Cytokeratin-19 (CK-19) is a low molecular weight cytokeratin filament responsible for the structural integrity of epithelial cells. It is highly expressed in PTC and only weakly or focally in FTC, mainly in the oncocytic variant. The expression of CK-19 is weak and focal in FTAs and negative in normal thyroid tissue. Some focal staining may appear near areas with chronic inflammation. CK-19 is commonly used in clinical diagnostics of thyroid nodules to identify conventional or FVPTC (106,123,126,146).

Hector Battifora Mesothelial cell (HBME-1), is an antibody targeted to microvilli on the surface of mesothelial cells. It is expressed strongly in PTCs and FTCs and weakly in FTAs.

It is absent or weakly positive in non-neoplastic thyroid tissue, such as nodular goitre or normal thyroid tissue, proposing a position as a neoplasia marker. Futhermore, upregulated expression of HBME-1 correlates with metastasis of thyroid cancer (53,106,127).

Transforming growth factor β (TGFβ) is an inhibitory growth factor that is involved in cell growth, differentiation and apoptosis. It causes an arrest in the G1 phase of the cell cycle. Resistance to TGF-mediated cell growth inhibition is a well-known pathogenesis in epithelial neoplasia. TGF receptor (TGFR) expression is shown to be reduced in minimally invasive FTCs compared to FTAs (128).

A new marker called insulin-like growth factor mRNA binding protein 3 (IMP-3), involved in cell proliferation and growth, has shown potential as a neoplasia marker. It is overexpressed in follicular-patterned thyroid carcinomas (FTC, FVPTC) and negative in benign thyroid tumours and normal thyroid tissue (129). In PDTCs, it is shown to have prognostic significance, correlating with distal and nodal metastatic disease and poorer overall survival (130).

5.4.6 Inducing angiogenesis

Cyclo-oxygenase 2 (COX-2) is involved in the synthesis of prostaglandins. It is also involved in tumorigenesis by promoting angiogenesis and inhibiting apoptosis. It is shown to be absent in normal thyroid tissue and hyperplastic nodules, but is expressed in FTA, PTC and FTC and thyroiditis (106). COX-2 expression is associated with poor prognosis in FTC (131), and in PTC (147).

Vascular endothelial growth factor (VEGF) stimulates migration and proliferation of endothelial vessels and promotes angiogenesis and vascular growth. In WDTC, increased VEGF expression is associated with metastasis and poor prognosis (127,132).

Epidermal growth factor (EGF) regulates cell growth, proliferation and angiogenesis. In addition, it opposes differentiation, by inhibiting thyroglobulin expression and iodide uptake. RAS-RAF-MEK-ERK and the AKT-PI3K pathways are known to be activated by EGFR. The amount of EGF receptors is increased in malignant thyroid tissue, especially in PDTCs and ATCs, which indicates a loss of TSH-dependency and inability to respond to radioiodine therapy (96,132,133). EGF is shown to activate invasion in FTC, by activating matrix metalloproteinase-9 (148).

Matrix metalloproteinases (MMP) participate in degrading extracellular matrix and are involved in tumour invasion and angiogenesis. MMP-1, -2 and -9 are shown to correlate with metastases in FTC (127,134).

5.4.7 Reprogramming of energy metabolism

Cancer cells use aerobic glycolysis (Warburg effect) for cell growth and survival. A link between sustained aerobic glycolysis and activation of oncogenes or loss of tumour suppressor genes is established (149). Specific changes in metabolic systems are linked to cancer. For example, mutations in fumarate hydratase correlate to kidney cancer and leimyomatosis, and mutations in succinate dehydrogenase to pheochromocytomas and paragangliomas. Mutated RAS gene, a gene involved in FTC carcinogenesis, is linked to enhanced glycolysis. Also mutations in PI3K, PTEN and TP53 genes, seen also in thyroid cancer, are linked to altered metabolism leading to increased metabolic function of cancer cells (149).

5.4.8 Inflammation and evading immune destruction

Inflammation has long been known to affect neoplastic lesions in an antagonizing way through innate immune cells (natural killer cells, cytotoxic T lymphocytes). On the other hand, chronic inflammation is shown to have tumour-enhancing effect. Chronic inflammation caused by microbes or various carcinogens is thought to initiate carcinogenesis rather than promote it. Interestingly, up to 15 to 20% of cancer cases worldwide are thought to be initiated by infections. Infection induces inflammation releasing e.g. reactive oxygen radicals which inflict genetic alterations and promote cancer formation (150,151). The association between inflammation and carcinogenesis has been demonstrated with Helicobacter pylori infection and stomach cancer (152), ulcerative colitis/ Crohn´s disease and colon cancer (153), human papilloma virus infection and cervical cancer (154), hepatitis C infection and liver cancer (155), as well as thyroiditis and thyroid cancer (156). On the other hand the presence of protecting immune cells, such as lymphocytes and dendritic cells, are connected to better survival (157).

Rearrangement of RET/PTC may link PTC and inflammation together. Activation of RET is shown in Hashimoto´s thyroiditis, which is a known risk factor for PTC (158-160). Interestingly, the oncogenic pathway of RAS gene and chronic inflammation are shown to cause tumour formation in a positive feedback manner (158,161). PI3K and MAPK pathways are also activated in thyroid carcinogenesis. They are also important in inflammation through activating chemokine receptors and promoting leukocyte migration (162).

The area adjacent to the tumour, where the inflammation often resides, is called the tumour microenvironment ("niche"). It contains multiple signals that enable the hallmarks of cancer to prevail. These chemotactic signals and growth factors affect cancer cells triggering EMT which is important in creating and maintaining CSCs, but also promotes tumour growth (EGF), angiogenesis (VEGF) and metastasis (MMPs). Tumours are portrayed as wounds that never heal, where tissue injury promotes cell proliferation and regeneration with the aid of inflammation but without a self-limiting trait (150,163).

Part of the immune system, such as natural killer cells or macrophages, acts to resist tumour formation and eliminates incipient cancer cells. Tumours able to evade this surveillance have managed to avoid the immunological recognition and the elimination of tumour cells, by being only weakly immunogenic or by inducing inflammation itself and recruiting own tumour-promoting cells of immune system, such as tumour-associated macrophages (TAMs). TAMs suppress the function of other immune cells thus securing tumour growth. Oncogene activation is shown to promote the formation of a tumour-promoting inflammation enhancing and improving the environment for tumour progression (151). On the other hand presence of immune cells in tumours, such as lymphocytes and dendritic cells, is connected to better survival. This may be explained by the tumour dormancy, i.e. tumour cells are inactive, but are activated if the surveillance of immune system is broken. This is exemplified with immuno-compromised individuals who often suffer from increasing occurrence of cancer formation due to loss of immune surveillance (102,164).

Transcription factor NF-κB, involved in innate immunity and inflammation, is activated by microbes, tissue damage, inflammatory cytokines and genetic alterations. It activates via toll-like receptors, which induce the TLR-MyD88 signaling pathway and eventually activate inflammatory mediators such as cytokines, adhesion molecules, proteases, angiogenic and antiapoptotic factors in order to promote cell survival. Indeed NF-κB is connected to tumour initiation and progression in association with inflammation (158).

Toll-like receptors are a family of transmembrane receptors that recognize conserved molecular patterns of microbial origin (pathogen associated molecular pattern, PAMP) but also endogenous ligands. They control the host defence from infection and are part of the first line defence mechanism by recruiting and stimulating both adaptive and innate immune responses. TLRs also have a role in tissue repair and tissue injury-involved inflammation by providing pro-survival and anti-apoptotic signals. They are shown to stimulate tumour cell proliferation and enhance survival (165). On the other hand, they also show an anti-tumour effect. For example TLR-7 and TLR-8 ligand, a drug called imiquimod, which is used to treat skin cancer (166) or TLR-9 ligand CpG is used to treat brain (167) and renal cancer (168). Thus the TLR system is considered a double-edged sword in cancer (169).

TLR-3, a receptor often seen in dendritic cells directed against viral antigens, is shown to be overexpressed in PTC cells, but not in FTC cells. It is associated with enhancing inflammatory reaction and cell growth in PTC (170). TLR-4, directed against lipopolysaccharides (LPS) of bacteria and viruses, is expressed in thyroid cells and it stimulates iodine uptake and thyroglobulin expression in them (171).

Cytokines, important mediators of inflammation produced by immune cells, are involved in activation, growth and differentiation of target cells, several of them having proinflammatory effects. In a study by Linkov et al (172) serum cytokines interleukin 8 (IL-8), hepatocyte growth factor (HGF), monocyte-induced interferon gamma (MIG), and IL-12p40 showed significant accuracy in differentiating between benign and malignant thyroid tumours. In another study, expression of cytokine CRCX4, stimulated by nitride oxide, was connected to

lymph node metastasis in PTC (173). A chemokine CCL21 activating chemokine receptor 7 stimulates tissue invasion and cell proliferation promoting thyroid carcinoma growth and lymph node metastasis (174).

Other mediators of inflammation have been detected in thyroid carcinomas such as previously mentioned TAMs (positive for CD68), which are shown to be abundant in PDTCs and ATCs compared to WDTCs. TAMs are correlated with capsular invasion, extrathyroidal extension and decreased cancer-related survival suggesting that TAMs may promote tumour progression (175).

Several tissue markers have been studied in follicular thyroid neoplasias to aid in the problematic field of their differential diagnostics. However, further studies are needed to identify clinically relevant immunohistochemical markers of neoplasia, markers of malignancy and markers of aggressiveness in FTC. The differentiation between a non-neoplastic and a neoplastic thyroid lesion is important, because recommended management of neoplastic thyroid lesions is surgery, whereas non-neoplastic lesions need only to be kept under surveillance. Understanding the path of thyroid carcinogenesis, helps to find markers for differentiating between benign and malignant thyroid lesions. Although FTC often acts indolently, it is important to distinguish those patients with an aggressive disease as early as possible to direct them to a more thorough management. The focus of my studies was pointed towards specific markers: MIB-1, Cyclin D1, p53, TLR-2, TLR-4, CD45, estrogen receptor alpha (ERα), ERβ, and a novel stem cell marker human embryonic stem cell 5 (HES5). I investigated their clinical utility in follicular thyroid neoplasias.

6 AIMS

The principal aim of this study was to find new markers for pre- and postoperative diagnosis of follicular thyroid neoplasia in order to minimize the need for diagnostic lobectomies, and to identify these cancer patients in need of more intensive follow-up and treatment.

The specific aims were:

1) to re-evaluate and study the role of demografic and histopathological features of FTCs on clinical outcome
2) to study the incidence of PDFTC in the HUCH region
3) to identify immunohistochemical
   - markers of neoplasia
   - markers of malignancy
   - markers of aggressiveness in FTC
   - prognostic markers of FTC

7 MATERIALS AND METHODS

7.1 Patient material

Follicular neoplasias were identified from the database of the Department of Pathology, HUCH (QPati database) from a time period between 1990 and 2009. In all, 398 follicular neoplasias were found, including 354 FTAs and 44 FTCs. Out of all follicular neoplasias, all FTCs were selected (n = 44), and atypical (n = 9) and oxyphilic (n = 29) adenomas were selected. A group of consecutive cases of typical FTAs (n=45) was selected. A total of 83 adenomas and thus 127 follicular neoplasias were included in the study. Futhermore, a non-neoplastic control tissue group of 86 specimens were selected, including 23 normal thyroid tissue samples, 41 nodular goitre samples and 22 hyperplastic thyroid tissue samples from patients with hyperthyroidism.

The total number of FTCs decreased by one from 44 patients, because the tissue material ran out (II, III). The staining of one FTA specimen failed during the study, decreasing the total number to 82 (III). In study I, the number of patients was smaller, consisting of 90 patients (39 carcinomas, 51 adenomas) from 1990 to 2006 with follow-up until March 2010.

7.2 Clinical data

Clinical data with follow-up extending until March 2011 was collected from patient records of the Department of Surgery and Oncology, Helsinki University Central Hospital (HUCH), survival data from the Population Register Center and cause of death from the patient records and from Statistics Finland. Collected patient data included age at the time of diagnosis, gender, symptoms prior to diagnosis, previous thyroid diseases, FNAB results if taken, TNM staging, surgery type (lobectomy or total thyreoidectomy), surgical complications, radioiodine treatments, administration of additional treatments (external radiation, chemotherapy and additional surgical treatments), recurrencies and secondary metastasis.

All FTCs diagnosed in HUCH region were gathered from a time period of 20 years from 1990 until 2009 (range 0-6 patients per year). Mean follow-up time for all FTC patients was 9.0 years. A mean survival time for all deceased patients (n=8) was 5.4 years, for PDFTC patients 4.7 years and for WDFTC patients 6.1 years. Altogether 83 adenomas were gathered as well as 86 non-neoplastic consecutive thyroid tissues. During follow-up, none of the adenoma patients developed thyroid cancer. Characteristics of study patients are shown in Table 7.

All study patients underwent thyroid surgery at the Department of Surgery, HUCH. Thyroid surgery was performed after a cytology report of a suspicion of a thyroid neoplasia (follicular neoplasia, oncocytic cells, suspicion of a PTC) to 30 of 44 (68%) patients with FTC, to 55

	All	Women	Men	Gender ratio
FTCs	44	24	20	1.2:1
Mean age (range)	58 yrs (24-83 yrs)	59 yrs (24-80 yrs)	56yrs (40-83 yrs)	
Mean size (range)	4.6 cm (0.6-14 cm)	4.5 cm (0.6-10 cm)	4.7 cm (1.2-14 cm)	
FTAs	83	66	17	3.9:1
Mean age (range)	49 yrs (19-79 yrs)	49 yrs (21-79 yrs)	48 yrs (19-69 yrs)	
Mean size (range)	2,5 cm (0.2-7 cm)	2.6 cm (0.2-7 cm)	2.1 cm (0.5-6 cm)	
Non-neoplasias	86	57	29	2.0:1
Mean age (range)	50 yrs (16-87 yrs)	47 yrs (18-87 yrs)	55 yrs (16-83 yrs)	
Normal thyroid tissue	23	16	7	2.3:1
Mean age (range)	41 yrs (19-63 yrs)	41 yrs (18-63 yrs)	42 yrs (29-59 yrs)	
Hyperplastic thyroid tissue	22	15	7	2.1:1
Mean age (range)	41 yrs (16-78 yrs)	39 yrs (25-60 yrs)	46 yrs (16-78 yrs)	
Goitrous thyroid tissue	41	26	15	1.7:1
Mean age (range)	60 yrs (25-87 yrs)	56 yrs (25-87 yrs)	65 yrs (42-83 yrs)	

Table 7. Clinical characteristics of study material.

of 83 (66%) patients with FTA. Surgery was performed due to an enlarged thyroid gland with cytology report indicating a goitrous lesion to 6 (14%) patients with FTC and to 16 (19%) patients with FTA. FNAB was skipped and primary surgery was proceeded due to a suspected large symptomatic goitre in 4 (9%) of FTC patients, in 9 (11%) of FTA patients; due to a suspected malignant disease (evident metastasis or widely invasive tumour to the surrounding tissues prior to surgery) in 4 (9%) of FTC patients; or due to hyperparathyreosis in 3 (4%) of FTA patients.

Lobectomy was performed in 30 of 44 (68%) FTC patients because suspicion of a malignancy was not evident in FNAB. In three (7%) patients a subtotal resection was done (istmectomy for one patient, resection of cervical lymph nodes for two patients). In all these 33 patients (75%) a completing thyroidectomy was performed after verification of a FTC at histology. Primary total thyroidectomy was performed in 11 (25%) FTC patients because of apparent metastases at the time (5 patients, 45%), a discovery of a grossly invasive growth of the tumour during operation (4 patients, 36%) or large multinodular thyroid with pressure symptoms (2 patients, 18%). Seven patients suffered from surgical complications: six from laryngeal nerve palsy and one from a hematoma of the neck.

All carcinoma patients received 30 – 120 mCi (1110 – 4440 MBq) radioiodine ablation (131I) within approximately 4 – 6 weeks after thyroidectomy, excluding one patient who

died 24 days after primary operation. Patients with advanced disease (n = 14, 32%) received additional radioiodine treatment, radiation, surgery or chemotherapy. All patients were followed until the end of March 2010, none were lost to follow-up.

7.3 Incidence data on thyroid cancer

Data concerning thyroid cancer incidence from 1970 until 2004 was received from Finnish Cancer Registry from courtesy of Professor Risto Sankila, M.D. This data, presented in study I, included incidence numbers as well as numbers of new cases group according to histology and gender in Finland.

7.4 Preparation of archived tissue material

The archival tumours were initially fixated into formalin in order to preserve the structure of the tissue and stop metabolism of the cells. Paraffin embedding was performed, after washing and dehydration with ascending series of alcohol solutions and diluting in an organic solvent, in order to harden the tissue to be cut into thin slides with a microtome. Archived paraffin blocks were cut with a microtome and paraffin was dissolved with a solvent and rehydrated with descending alcohol series. Haematoxylin stain was performed in water and counterstained with eosin in alcohol after dehydration. Finally, the stained slides were mounted with a non-aqueous medium and a coverslip. Haematoxylin stains the cytoplasm of the cell into pinkish colour whereas eosin stains nuclei as blue (HE stain) (176).

7.5 Re-evalution of histology

HE stained tissue slides were re-evaluated using a light microscope (Leica DMLB microscope, Meyer Instruments Inc, Houston, Texas, USA) and classified according to WHO classification by two pathologists (Johanna Arola and Jaana Hagström) (2).

7.5.1 Adenomatous or goitrous nodule

Multiple nodules lacking a well-defined capsule with no difference from the surrounding normal thyroid tissue´s were classified as goitrous nodules.

7.5.2 Atypical adenoma

A highly cellular tumour with evidence of increased proliferative activity as well as cellular and nuclear atypia, but lacking invasion to the capsule or a vessel, was classified as atypical adenoma. (Figure 5A, B)

7.5.3 Oxyphilia

A tumour was considered oxyphilic if it was solely or predominantly (≥75%) composed of oxyphilic cells. Oxyphilic cells were loosely cohesive with a moderate amount of deeply eosinophilic and granular cytoplasm and round nuclei. Growth pattern was either solid or trabecular with low amount of colloid present. Nuclei were large, hyperchromatic and pleomorphic with a prominent nucleoli. (Figure 5C)

7.5.4 Invasiveness

Invasive growth was required for the diagnosis of FTC. Invasive growth included vascular and capsular invasion. Vascular invasion was verified within the vessels of the capsule or outside it, but not in the vessels inside the tumour. Either an endothelium-surrounded tumour cells as a thrombus or a penetrating mass indicated a vascular invasion. Capsular invasion was comprehended as a penetration of the tumour tissue through the capsule. It was distinguished from the penetration wound of FNAB (Figure 5D-G). Minimally invasive FTCs exhibited only focal invasion either to the capsule or a vessel, whereas widely invasive FTCs showed widespread infiltration into adjacent thyroid tissue or into vessels.

7.5.5 Poor differentiation

PDFTCs were separated from well-differentiated FTCs (WDFTCs), with the former showing thyroglobulin positive tumour cells with a high proliferative activity (MIB-1 / PI index of 10-30%). In PDFTCs, growth pattern was infiltrative with either solid, trabecular or insular appearance. Foci of necrosis and mitoses were frequently present and vascular invasion was obvious. Tumour cells were dyscohesive and irregularly sized and only scant colloid was present. Nuclei were bland and usually monomorphic with fine chromatin and small nucleoli (Figure 5H, I).

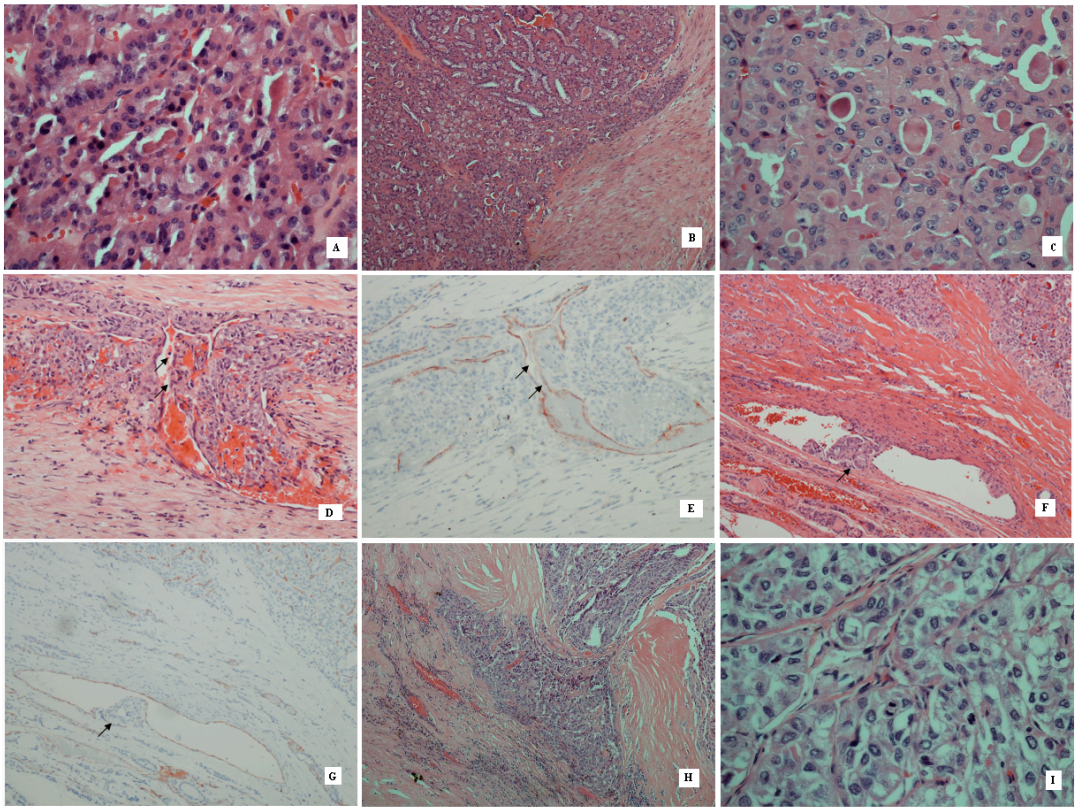


Figure 5. A and B: HE stainings of atypical adenomas, original magnifications of 40X and 10X. C: HE-staining of oxyphilic adenoma, original magnification 40X. D and F: HE-stainings showing an obvious vascular invasion with a penetrative manner (D) as well as a thrombus inside the vessel (F). Original magnifications 10X. E and G: CD31 staining verifies the presence of endothelium in invasions (figure D corresponding to E and figure F to G). Original magnifications 10X. H and I: HE-staining of PDFTCs. Original magnifications of 10X and 40X.

7.6 Process of immunohistochemistry

Through immunohistochemical staining procedures a specific epitope of an antigen can be detected in a tissue specimen. These epitopes often need retrieval for exposing the immunogenic sites from cross-linking proteins and thus increasing the sensitivity of the method. In this study, the retrieval was performed using a pretreatment (PT) module of an Autostainer (LabVision UK, Ltd., Cheshire, UK) with a trishydroxymethylaminomethane hydrochloride (tris-HCl) buffer or heated in a microwave in a citrate buffer. A peroxidase-blocking solution (methanol and hydrogen peroxidase) was used to destroy the endogenous peroxidase activity. Normal serum was used to block nonspecific staining. For antibody binding, we used the avidin-biotin complex (ABC) method. This method involves three layers: the first layer is the unlabelled primary antibody, the second consists of the biotinylated secondary antibody (immunoglobulin G (IgG)) and the third is a complex of avidin-biotin

peroxidase or horseradish peroxidase (HRP), which is finally bound with a specific substrate (3,3'-diaminobenzidine [DAB] or 3-amino-9-ethylcarbazole [AEC]) to produce a specific coloring of the specimen. Counterstaining was performed with Meyer´shaematoxylin.

An antibody can be either monoclonal (mAb) or polyclonal (pAb). The mAbs are manufactured in cells that are identical to each other with a monovalent affinity of binding to a single epitope. They are produced with a hybridoma technique by fusing myeloma cells with B cells from the spleen of a mouse that has been immunized with the antigen desired. On the other hand, pAbs are obtained from different B cells identifying different epitopes of a specific antigen. The mAbs are more expensive, more time-consuming and more difficult to produce than pAbs. The mAbs are renewable, whereas pAbs are not. The pAbs can amplify a low expression level of a target protein. Futhermore, they can be directed against an unknown antigen. However, they suffer from background signaling and cross-reactivity (177).

7.7 Tissue microarray

For immunohistochemistry, a tissue microarray (TMA) block was constructed (II, III). Tumour core punctures were taken from two representative areas of the original paraffin tumour blocks: one from the border and one from the central area of the tumour. Maximum of 56 cores were inserted into a recipient paraffin block using a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA, MTABooster® Version 1.01 for Beecher Manual Arrayer, Alphelys). Three sections from different levels were cut from each TMA block to obtain nine spots per tissue sample for immunohistochemical analysis. (Figure 6).

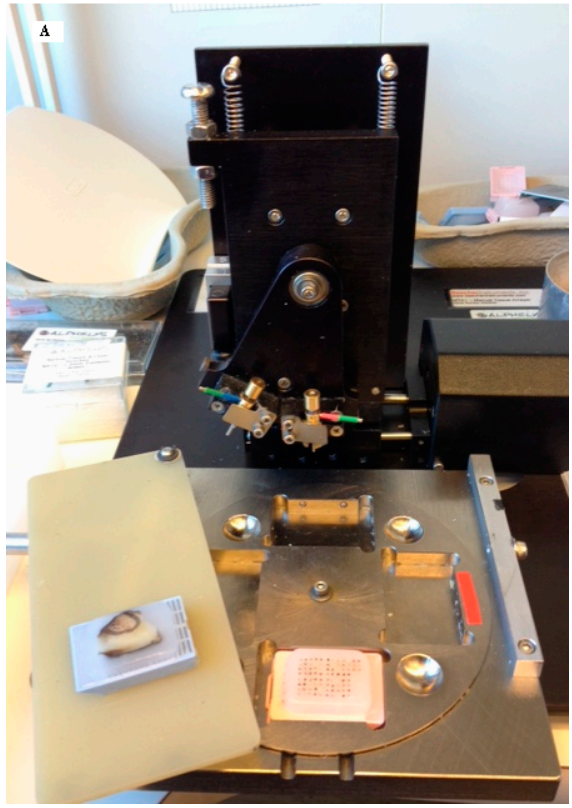


Figure 6. Tissue microarrayer. Beecher Manual Arrayer, Alphelys.

7.8 Immunohistochemistry

Whole sections (I, IV) and TMA sections (II, III) were used. Formalin-fixed and paraffin-embedded tumour samples were cut into 4-nm sections and heated in 55°C for 2 hours. Slides were deparaffinized in xylene, and rehydrated through graded alcohol series and destilled water and treated in a the PT of an Autostainer (LabVision) in either Tris–HCl buffer (pH 8.5) or Tris-ethylenediaminetetraacetic (EDTA) buffer (pH 9.0) for 20 min at 98°C. Staining was performed in an Autostainer 480 (LabVision), using the Dako REAL EnVision Detection System, Peroxidase/DAB+, rabbit/mouse (Dako, Glostrup, Denmark). Before immunohistochemical staining, the slides were treated with 0.3% Dako REAL peroxidase-blocking solution for 5 min following incubation with a specific antibody (Table 7) in correct dilution for 1 h, using Dako REAL antibody diluent, followed by a 30-min incubation with Dako REAL EnVision/HRP detection system, Rabbit/Mouse (ENV) reagent. Visualization was carried out by Dako REAL DAB+ Chromogen for 10 min. The slides were washed with phosphate-buffered saline (PBS)-0.04%Tween20 between each step. Counterstaining was performed with Meyer's haematoxylin, followed by washing in tap water for 10 min and mounted with mounting medium (Aquamount, BDH, Poole, UK). The immunohistochemical staining procedure was performed similarly in each study, except for platelet endothelial cell adhesion molecule 1 (CD31/PECAM1).

The immunostaining with CD31 antibody was done manually, using whole sections. After deparaffination and dehydration, the slides were treated in a microwave oven in citrate buffer (pH 6.0) and then incubated in methanol and hydrogen peroxidase. A Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) was used according to manufacturer´s instructions for immunostaining with CD31 antibody. AEC containing hydrogen peroxide was used for visualization of the antibody and Meyer's haematoxylin for counterstaining. The slides were mounted with aqueous mounting media (glycerol vinyl alcohol aqueous mounting solution; Zymed Laboratories, South San Francisco, CA, USA). A negative control for staining was received by omitting the primary antibodies during the procedure. Thyroglobulin staining was performed to ensure the correct origin of the tumour as well as its viability.

7.9 Antibodies

A descriptive list of antibodies used (I-IV) are shown in Table 8. A novel monoclonal antibody named HES5 was used (II). It was produced by a collaborating laboratory (Christian Fermér and Olle Nilsson, Fujirebio Diagnostics AB). An undifferentiated human embryonic (hES) stem cell line SA167 (Cellartis, Göteborg, Sweden) was used for immunization of the mice. Conventional hybridoma technology (178) was used to establish hybridoma cell lines producing monoclonal antibodies (mAbs) against hES cells. The detailed description of the method is in the supplementary data of the study II.

Antigen	Study	Antibody	Antibody type	Manufacturer	Dilution	Staining type
Cyclin D1	I	SP4	rabbit mAb	Neomark	1:20	Nuclear
p53	I	DO-7	mouse mAb	Dako	1:150	Nuclear
Ki-67	I, III	MIB-1	mouse mAb	Dako	1:150	Nuclear
PECAM-1	I	CD31	mouse mAb	Dako	1:100	Endothelium
TLR2	IV	sc-10739	rabbit pAb	Santa Cruz	1:50	Cytoplasmic
TLR4	IV	sc-10741	rabbit pAb	Santa Cruz	1:50	Cytoplasmic
T200, Ly-5	II	CD45	mouse mAb	Dako	1:750	Cytoplasmic
ERα	III	NCL-L-ER-6F11	mouse mAb	Novocastra	1:50	Nuclear
ERβ	III	sc-8974	rabbit pAb	Santa Cruz	1:100	Nuclear
Thyroglobulin	III	Anti-human Tg	rabbit pAb	Dako	1:2000	Cytoplasmic
unknown	II	HES5	mouse mAb	noncommercial	1:300	Nuclear Cytoplasmic

Table 8. Overview of antibodies in the study.

Immunohistochemical staining

Two independent researchers (Annukka Heikkilä and Jaana Hagström) scored tumour specimens. The researchers did not know the clinical status. Different scoring categories were used for different immunohistochemical stains (Table 8, Figure 7).

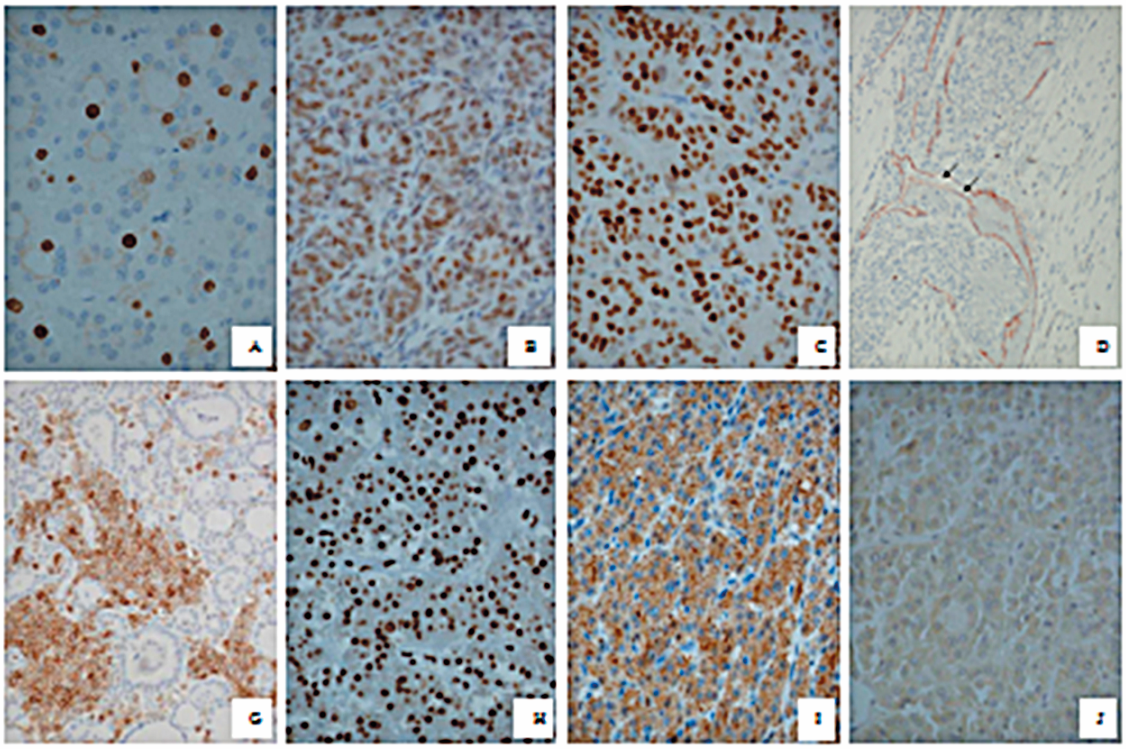
Whole-tissue specimens were stained with MIB-1 antibody (I, IV). The nuclei were stained and scored as<2%, 2–5%, 5–10% and >10%. Tonsilla tissue was used as positive control tissue.

The p53 antigen was also used to stain whole-tissue sections (I). The nuclei were stained and scored as negative, 1–10%, 10–50% and >50%. Colon tissue was used as a positive control.

Futhermore, whole-tissue section were stained with Cyclin D1 (I). Nuclear staining was observed. The samples were scored as negative, 1–10%, 10–50% and >50%. Tissue from the ventricle was used as a positive control.

CD31 was used as a marker for endothelium surrounding the vessels to identify vascular invasion (I). Whole-tissue sections were used. Scoring was either positive or negative.

A TMA construction was used and recipient slides were stained with HES5 antibody (II). Expression in cytoplasmic staining was scored as none, mild, moderate or strong, according to its intensity. Nuclear staining was scored by the percentage of positively stained nuclei, namely zero, low (1–35%), medium (36–75%) or high (>75%). Colon tissue was used as a positive control.



The expression of ERα and ERβ was studied, using the TMA method (III). The immunohistochemical stainings were nuclear and were scored by the percentage of positive nuclei with a 5% accuracy. Mammary tissue was used as a positive control.

TLR-2 and TLR-4 expression was verified from whole-tissue sections (IV). The staining was cytoplasmic, with intensity of the expression scored as none, mild, moderate or strong.

CD45 from whole-tissue sections was scored as negative when none or only a few lymphocytes were present and positive when lymphocyte accumulations were seen (IV). Infected gingival tissue was used as the positive control.

Thyroglobulin staining was scored as either negative or positive in identifying thyroid tissue. Omission of primary antibodies served as a negative control with each staining.

7.10 Statistical analysis

The associations between the various immunohistochemical stainings and clinicopathological variables were assessed by the chi-square test and Fisher´s exact test when number of cases was

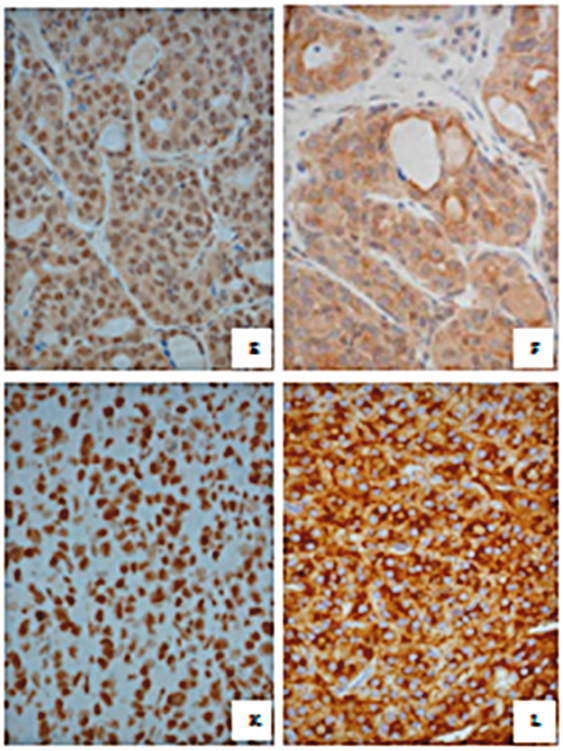


Figure 7. Panel of all immunohistochemical markers studied. A. MIB-1, B. p53, C. Cyclin D1, D. CD31, arrows indicating an invasion of the tumour cells to a vessel, E. TLR-2, F. TLR-4, G. abundant CD45-positive lymphocytes, H. HES5 nuclear positivity, I. HES5 cytoplasmic positivity, J. ERα, K. ERβ, L. thyroglobulin positivity.

insufficient. Survival time was calculated starting from date of the primary surgery to either last day of follow-up or either death due to other causes (censored cases) or cancer-related death, with survival curves constructed using the Kaplan-Meier method and compared with the log rank test. A p-value below 0.05 was confined as statistically significant. Receiver – operating characteristic (ROC) curve analysis was used to compare the diagnostic value of HES5 to calculate the area under the curve (AUC) value. The logistic regression model was used to evaluate whether two markers provided independent diagnostic information and whether their combination would improve accuracy. Statistical analyses were performed using SPSS 15.0 – 17.0 software (SPSS Inc., Chicago, IL, USA / IBM – SPSS, Armonk, NY, USA).

7.11 Study ethics

The Surgical Ethics Comittee of HUCH and National Supervisory Authority of Welfare and Health (Valvira) approved the gathering of data from the Population Register Centre and the Hospital Discharge Registry. Statistics Finland gave permission to use the Cause of the Death Register. No deterioration of disease status of patients was detected during the studies (I-IV).

8 RESULTS

8.1 Clinical and histological characteristics of follicular neoplasias

The data on thyroid cancer incidence in Finland was received from Finnish Cancer Registry showing that during the past 30 years, the incidence of thyroid cancer has increased. The increase is due to an increase in the incidence of PTC cases, while FTC cases have decreased (Figure 8). This decrease was more evident in female patients, while the yearly number of new male patients remained nearly constant.

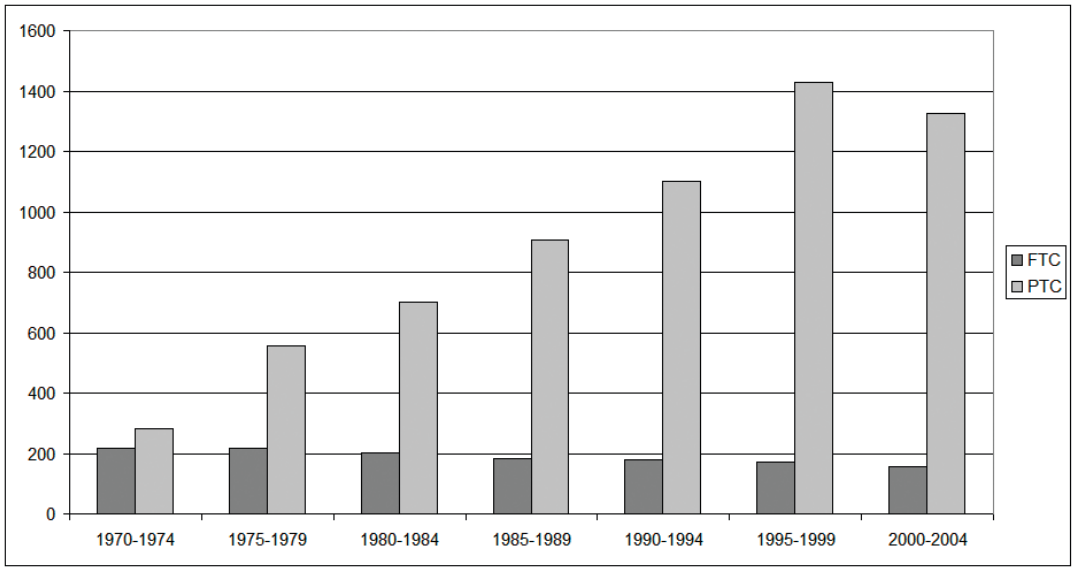


Figure 8. Number of new PTC and FTC patients from 1970 to 2004 in Finland (data from Professor Risto Sankila, Finnish Cancer Registry).

The study material of follicular neoplasias diagnosed in the HUCH region was gathered over a time period of 20 years from 1990 until 2009. The material included 44 FTC specimens, of which 37 (84%) were WDFTCs (mean age 56 years, 60 years for women, 55 years for men) and 7 (16%) were PDFTCs (mean age 59 years, 53 years for women, 61 years for men) (Figure 9). The gender ratio for WDFTCs was 1.5:1 (22 women, 15 men) and for PDFTCs 1:2.5 (2 women, 5 men). Of the WDFTCs, 10 (27%) were minimally invasive and 27 (73%) were widely invasive, whereas all 7 PDFTCs were widely invasive. Oxyphilia occurred in 15 (34%) FTCs, two (2 of 7, 29%) of which were PDFTCs and the last 13 were WDFTCs (13 of 37, 35%). Patients with minimally invasive disease (n=10, 23%) were free from metastasis and residives throughout the entire follow-up.

A cohort of 20 (45%) FTC patients with aggressive disease with primary or secondary, regional or distant metastases were selected. This group included all PDFTCs (n=7) as well as 13 WDFTCs with widely invasive growth patterns. Of 44 FTC patients, 7 (16%) developed primary metastasis and additional 7 developed secondary metastases. Recurrences emerged in 13 (30%) patients. Five patients showed nodal invasion at the time of diagnosis, and each had at least a T3 tumour of TNM staging (one T3, four T4, p=0.043, unpublished data). All carcinoma patients with primary distant metastasis (n=7) had tumours at least 4 cm in diameter, the average size being 7.6 cm. Size, age (both with various cutoff values) or gender did not correlate with the invasiveness of the tumour (minimally versus widely), nor with metastasis, differentiation level of the tumour or survival of all FTC patients (unpublished data).

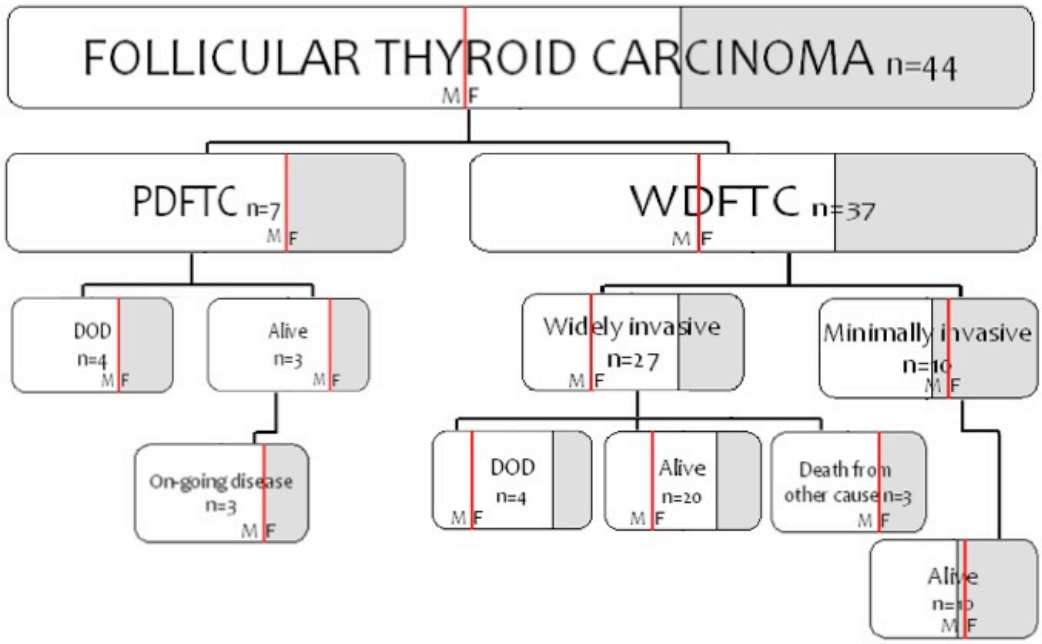


Figure 9. Introducing HUCH region follicular thyroid carcinoma study material between 1990 to 2009 in flow charts. Grey portion of boxes indicates the proportion of oxyphilic tumours in the group. Red line indicates the gender ratio (M = male, F = female, DOD = death of disease).

In all, 354 adenoma patients were diagnosed during the study period in the HUCH region, of which 287 were women and 67 were men (gender ratio 4.3:1). A total of 83 adenomas (66 women and 17 men, gender ratio 3.9:1) were included in the study. A consecutive adenoma cohort was selected, consisting of typical adenoma patients (n=45, 36 women and 9 men, gender ratio 4:1). Each atypical (n = 9, 7 women and 2 men, gender ratio 3.5:1) and oxyphilic (n = 29, 23 women and 6 men, gender ratio 3.8:1) adenoma specimen was selected. Of all FTAs diagnosed in the HUCH region, oxyphilia occurred in 8% (29 of 354) (Figure 10).

Oxyphilia was statistically more common in FTCs than in FTAs when the entire HUCH population was acknowledged from 1990 to 2009 ($p<0.001$, unpublished data). The mean size of all FTA tumours was 2.5 cm (range 0.2–7 cm), in women 2.6 cm (range 0.2–7 cm) and in men 2.1 cm (range 0.5–6 cm). The FTAs were significantly smaller than carcinomas ($p<0.001$, unpublished data).

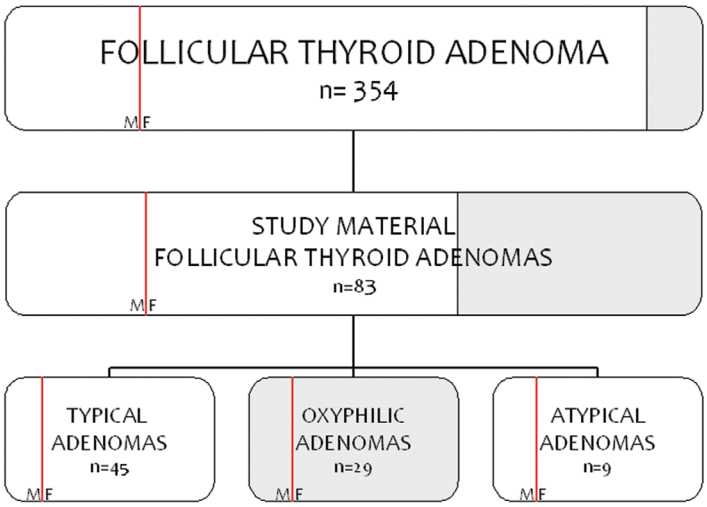


Figure 10. Introducing HUCH region follicular thyroid adenoma study material between 1990 to 2009 in flow charts. Grey portion of boxes indicates the proportion of oxyphilic tumours in the group. Red line indicates the gender ratio (M=male, F=female).

8.2 Treatment of follicular thyroid neoplasias

All study patients underwent thyroid surgery at the Department of Surgery, HUCH. Surgical removal of the gland was performed conservatively, with minimal damage to adjacent tissues, including the laryngeal nerves and parathyroid glands. All carcinoma patients received radioiodine ablation after thyroidectomy, excluding one patient. Patients with advanced disease received additional radioiodine treatment, radiation, surgery or chemotherapy. The details of specific treatments are seen in Table 9.

Pat no	Age	Sex	Oxy	Diff.	Vasc	Caps	Necr	Invas	Size (cm)	T	N	M	Stage	MIB-1	primM	secM
1	62	F	No	WDFTC	Yes	Yes	No	Wide	7	4	0	1	4	0-2%	Trachea, pleura	No
2	50	M	Yes	**PDFTC**	Yes	Yes	No	Wide	8	4	1	1	4	25 %	Lungs	No
3	73	F	No	**PDFTC**	Yes	Yes	No	Wide	10	4	1	0	4	10 %	No	Lungs
4	57	F	No	WDFTC	Yes	Yes	No	Wide	4	2	0	1	4	0-2%	Bone	No
5	77	M	No	**PDFTC**	Yes	Yes	Yes	Wide	2,5	2	0	0	2	15 %	No	Bone
6	54	M	Yes	WDFTC	Yes	Yes	No	Wide	8	4	1	0	4	5-10%	No	Bone
7	61	M	No	**PDFTC**	Yes	Yes	Yes	Wide	4	4	0	0	4	20 %	No	Lungs, liver
8	67	F	No	WDFTC	Yes	Yes	Yes	Wide	8	3	0	1	4	0-2%	Bone	No
9	47	M	No	**PDFTC**	Yes	Yes	Yes	Wide	14	4	1	1	4	15 %	Mediastinum, vena cava	Bone, lungs
10	72	M	Yes	**PDFTC**	Yes	Yes	No	Wide	5	3	0	0	3	15 %	No	Lungs
11	33	F	No	**PDFTC**	Yes	Yes	No	Wide	2	1	0	0	1	30 %	No	Lungs, brain
12	49	M	No	WDFTC	No	Yes	No	Wide	4	2	0	0	2	2-5%	No	Lungs, bone
13	40	M	No	WDFTC	No	Yes	No	Wide	5	3	0	1	2	0-2%	Brain	No
14	52	F	Yes	WDFTC	Yes	Yes	No	Wide	2	1	0	0	1	2-5%	No	No
15	72	F	No	WDFTC	Yes	Yes	No	Wide	7	3	0	0	3	2-5%	No	No
16	52	M	Yes	WDFTC	Yes	Yes	No	Mini	2	1	0	0	1	5-10%	No	No
17	56	M	No	WDFTC	No	Yes	No	Mini	3	3	0	0	3	0-2%	No	No
18	40	F	No	WDFTC	Yes	Yes	No	Wide	2	1	0	0	1	0-2%	No	No
19	45	M	No	WDFTC	Yes	Yes	No	Wide	4	2	0	0	2	5-10%	No	No
20	76	F	Yes	WDFTC	No	Yes	No	Wide	3	2	0	0	2	5-10%	No	No
21	48	M	No	WDFTC	No	Yes	No	Wide	6	3	0	0	3	2-5%	No	No
22	63	F	No	WDFTC	No	Yes	No	Mini	4	2	0	0	2	0-2%	No	No
23	50	F	No	WDFTC	No	Yes	No	Mini	0,6	1	0	0	1	0-2%	No	No
24	69	F	No	WDFTC	Yes	Yes	No	Wide	8	4	0	1	4	2-5%	Lungs	No
25	24	F	No	WDFTC	Yes	Yes	No	Wide	5	3	0	0	1	5-10%	No	No
26	76	F	Yes	WDFTC	Yes	No	No	Mini	3	2	0	0	2	5-10%	No	No
27	71	F	No	WDFTC	Yes	Yes	Yes	Wide	8	4	0	0	4	2-5%	No	No
28	60	F	Yes	WDFTC	Yes	Yes	No	Wide	5	3	0	0	3	2-5%	No	No
29	67	M	Yes	WDFTC	Yes	Yes	No	Wide	3	2	0	0	2	2-5%	No	No
30	35	F	No	WDFTC	No	Yes	No	Wide	3	2	0	0	1	2-5%	No	No
31	74	F	No	WDFTC	Yes	Yes	No	Wide	4	2	0	0	2	2-5%	No	No
32	56	M	No	WDFTC	Yes	Yes	No	Wide	3	2	0	0	2	0-2%	No	No
33	80	F	No	WDFTC	Yes	Yes	No	Wide	8	3	0	0	3	2-5%	No	No
34	43	M	No	WDFTC	Yes	Yes	No	Mini	5	3	0	0	1	0-2%	No	No
35	71	F	Yes	WDFTC	Yes	Yes	No	Wide	1	1	0	0	1	5-10%	No	No
36	48	F	Yes	WDFTC	Yes	Yes	No	Wide	2,5	2	0	0	2	5-10%	No	No
37	54	M	No	WDFTC	No	Yes	No	Mini	1	1	0	0	1	0-2%	No	No
38	55	M	Yes	WDFTC	Yes	Yes	No	Mini	3	2	0	0	2	2-5%	No	No
39	78	F	No	WDFTC	Yes	Yes	Yes	Wide	6	3	0	0	3	5-10%	No	No
40	47	M	Yes	WDFTC	Yes	No	Yes	Mini	3	2	0	0	2	2-5%	No	No
41	47	F	Yes	WDFTC	Yes	No	No	Mini	4	2	0	0	2	0-2%	No	No
42	83	M	No	WDFTC	No	Yes	No	Wide	6	3	0	0	3	2-5%	No	No
43	45	F	No	WDFTC	Yes	No	No	Wide	2	1	0	0	1	5-10%	No	No
44	69	M	Yes	WDFTC	Yes	Yes	No	Wide	5	3	1	0	4	2-5%	No	No

RaI (n)	RaI Total (mCi)	Rad (n)	Chemo	Second. Surg.	Follow -up (yrs)	Status
0	No	0	No	No	0.1	DOD
1	100	1	No	No	0.6	DOD
3	380	1	No	No	3.2	DOD
5	730	3	No	No	4.2	DOD
3	330	3	No	No	5.5	DOD
4	470	3	ADR	M colli, trachea	7.4	DOD
2	180	1	ADR, Gem	No	9.4	DOD
4	550	3	No	No	12.6	DOD
3	370	3	ADR	No	2.7	On-going
5	460	1	No	M colli	4.1	On-going
4	520	4	ADR, Nexavar	M pulmonum cranii	6.5	On-going
5	750	6	No	M femoris	17.8	On-going
2	200	0	No	M cerebri	11.4	Free
1	100	0	No	No	14.7	Free
2	250	0	No	No	18	Free
2	230	0	No	No	18.3	Free
2	250	0	No	No	19.3	Free
1	100	0	No	No	19.7	Free
5	730	1	No	No	19.9	Free
1	120	0	No	No	20	Free
1	100	0	No	No	20	Free
2	230	0	No	No	20.5	Free
1	100	0	No	No	20.6	Free
4	450	0	No	No	2.5	Free
2	200	0	No	No	3.5	Free
1	30	0	No	No	3.8	Free
2	150	1	No	No	4.5	Free
1	100	0	No	No	4.5	Free
5	350	0	No	No	4.8	Free
1	30	0	No	No	5.3	Free
1	60	0	No	No	5.5	Free
1	100	0	No	No	5.5	Free
2	180	0	No	No	5.7	Other
1	100	0	No	No	6	Free
1	60	0	No	No	6.8	Free
1	30	0	No	No	7	Free
1	100	0	No	No	7	Free
1	100	0	No	No	7.3	Free
2	165	0	No	No	7.5	Free
1	30	0	No	No	8.3	Free
1	30	0	No	No	8.4	Free
2	180	0	No	No	9.7	Other
1	100	0	No	No	10.9	Free
2	250	1	No	No	16.1	Other

Table 9. Therapies of all FTC patients in chronological order according to follow-up time within status groups. Abbreviations: **Pat no** = patient number, **Oxy** = oxyphilia, **Diff** = differentiation type, WDFTC = well-differentiated follicular thyroid carcinoma, PDFTC = poorly differentiated follicular thyroid carcinoma, **Vasc** = vascular invasion, **Caps** = capsular invasion, **Necr** = necrosis, **Invas** = invasiveness, **Wide** = widely invasive, **Mini** = minimally invasive, **T** = tumour = size of tumour, **N** = nodus = lymph node metastasis, **M** = distal metastasis, **PrimM** = primary metastasis, **SecM** = secondary metastasis, **RaI** = radioiodine treatment, **Rad** = radiation therapy, **Chemo** = chemotherapy, **ADR** = Adriamycin, **Gem** = Gemcitabin, **Second surg** = secondary surgery, **DOD** = death of disease, **On-going** = on-going disease, **Other** = other cause of death.

8.3 Immunohistochemistry

8.3.1 Non-neoplastic lesions

A novel monoclonal stem cell marker HES5 was studied and a significant correlation was found, with expression differing between non-neoplastic, e.g. goitrous, hyperplastic and normal thyroid tissue, and neoplastic follicular thyroid tissue (II). Nuclear expression of HES5 was more marked in non-neoplastic lesions, whereas cytoplasmic expression was stronger in neoplastic lesions (Table 10). Nuclear expression was a more sensitive and specific trait than cytoplasmic staining in predicting the likelihood of a neoplasia. Using ROC curve analysis, the AUC value was higher for nuclear staining than for cytoplasmic staining (AUCnucleus=0.752, 95% confidence interval (CI) 0.686–0.818 versus AUCcytoplasm=0.681, 95% CI 0.608–0.754).

HES 5 – amount of nuclei stained, n (%)	**0 %**	**1–35%**	**36–75%**	**>75%**
all FTC (n=42)	29 (69)	4 (10)	3 (7)	6 (14)
all FTA (n=82)	46 (56)	19 (23)	7 (9)	10 (12)
goitre (n=41)	4 (10)	15 (37)	13 (32)	9 (22)
hyperplasia (n=22)	3 (14)	8 (36)	8 (36)	3 (14)
normal (n=23)	2 (9)	11 (48)	8 (35)	2 (9)

HES 5 – intensity of cytoplasmic staining, n (%)	**zero**	**mild**	**moderate**	**strong**
all FTC (n=42)	2 (5)	12 (29)	15 (36)	13 (31)
all FTA (n=82)	3 (4)	31 (38)	29 (35)	19 (23)
goitre (n=41)	0 (0)	22 (54)	19 (46)	0 (0)
hyperplasia (n=22)	1 (5)	19 (86)	2 (10)	0 (0)
normal (n=23)	0 (0)	21 (91)	1 (4)	1 (4)

Table 10. Expression of HES5 in study material.

8.3.2 Neoplastic lesions

Several markers in our studies differentiated between FTA and FTC. Of 44 FTCs and 83 FTAs, a significant correlation was detected with MIB-1/Ki-67 expression, showing a higher expression in FTCs ($p<0.001$, unpublished data).

Cyclin D1 expression was higher in FTCs than in FTAs (I, $p=0.001$). The expression showed no significant correlation with clinical parameters nor with survival ($p=0.640$, unpublished data) (Table 11).

ERβ expression differed significantly between FTAs and FTCs, with FTAs exhibiting a stronger expression (II) (Table 11). Logistic regression analysis showed that ERβ is more sensitive, with an odds ratio of 0.85 (95% CI 0.79–0.92, $p<0.001$), as a differential marker between FTA and FTC than MIB-1 ($p=0.018$). ROC curve analysis showed that the diagnostic value of ERβ is again stronger than that of MIB-1 (AUC 0.757 versus 0.677) in our material (III). Combining ERβ and MIB-1 expressions resulted in no additional benefit for differential diagnosis, as calculated with a cancer probability index. No significant correlation was detected between genders with ERα or β expressions (unpublished data).

A difference in expression between benign and malignant tissues with TLR-2 was shown (IV), with a stronger expression in adenomas ($p=0.013$), although this marker showed no significant correlation with other significant clinical parameters (Table 11).

MIB-1, n(%)	**0−2%**	**2−5%**	**5−10%**	**>10%**
FTC (n=44)	12(27)	15(34)	10(23)	7(16)
FTA (n=83)	43(52)	26(31)	14(17)	0(0)
Cyclin D1, n(%)	**0−10%**	**10−50%**	**50−80%**	**80−100%**
FTC (n=39)	13(33)	20(51)	6(15)	0(0)
FTA (n=51)	21(41)	25(41)	5(10)	0(0)
p53, n(%)	**0−10%**	**10−50%**	**50−80%**	**80−100%**
FTC (n=39)	34(87)	4(10)	1(3)	0(0)
FTA (n=51)	45(88)	4(8)	2(4)	0(0)
TLR-2, n(%)	**none**	**mild**	**moderate**	**strong**
FTC (n=44)	1(2)	30(68)	13(30)	0(0)
FTA (n=83)	1(1)	38(46)	35(42)	9(11)
TLR-4, n(%)	**none**	**mild**	**moderate**	**strong**
FTC (n=44)	3(7)	21(48)	14(32)	6(14)
FTA (n=83)	1(1)	30(36)	33(40)	19(23)
ERα, n(%)	**0%**	**<1%**	**5%**	**≥10%**
FTC (n=43)	40(93)	2(5)	0(0)	1(2)
FTA (n=82)	73(89)	9(11)	0(0)	0(0)
ERβ, n(%)	**0%**	**<1%**	**5%**	**≥10%**
FTC (n=42)	4(10)	7(17)	6(14)	25(60)
FTA (n=82)	1(1)	3(4)	2(2)	76(93)

Table 11. Expression of immunohistochemical markers used (I, III and IV).

8.3.3 Aggressive follicular thyroid carcinomas

PDFTCs showed a higher MIB-1 expression/PI than WDFTCs (p<0.001) and metastatic tumours (primary and/or secondary, p=0.001), while aggressively acting tumours also showed higher PI (p=0.021) (I). MIB-1 showed no correlation with survival (p=0.094) when the original scoring pattern was used. However, when group from 0% to 5% was combined, a statistically significant correlation was achieved between MIB-1 expression and survival (p=0.007, Figure 11, unpublished data). With a cutoff point of 10%, e.g. between WDFTC and PDFTC, a stronger correlation was found (p<0.001, unpublished data) with patients with high PI having a 5-year survival of 69% versus those with a low PI 94%.

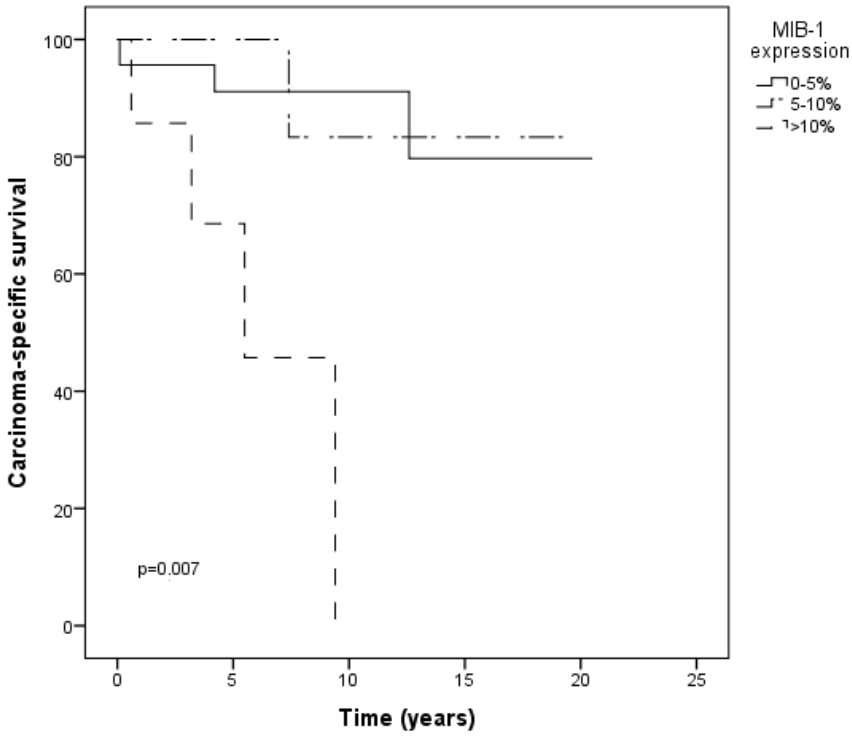


Figure 11. Kaplan-Meier survival figure showing a correlation between high MIB-1 expression and poor survival.

I failed to detect any correlation with the tumour material examined and p53 expression, because the majority of all neoplasias studied showed negative or mild expression, even in the PDFTCs (I). No prognostic correlation was achieved with p53 expression (p=0.903, unpublished data).

The data concerning patients´ stimulated serum thyroglobulin levels prior to the first radio ablation was gathered and a significant correlation was found between high levels of thyroglobulin and poor survival (unpublished data, Figure 12). Patients with antithyroglobulin antibodies were excluded.

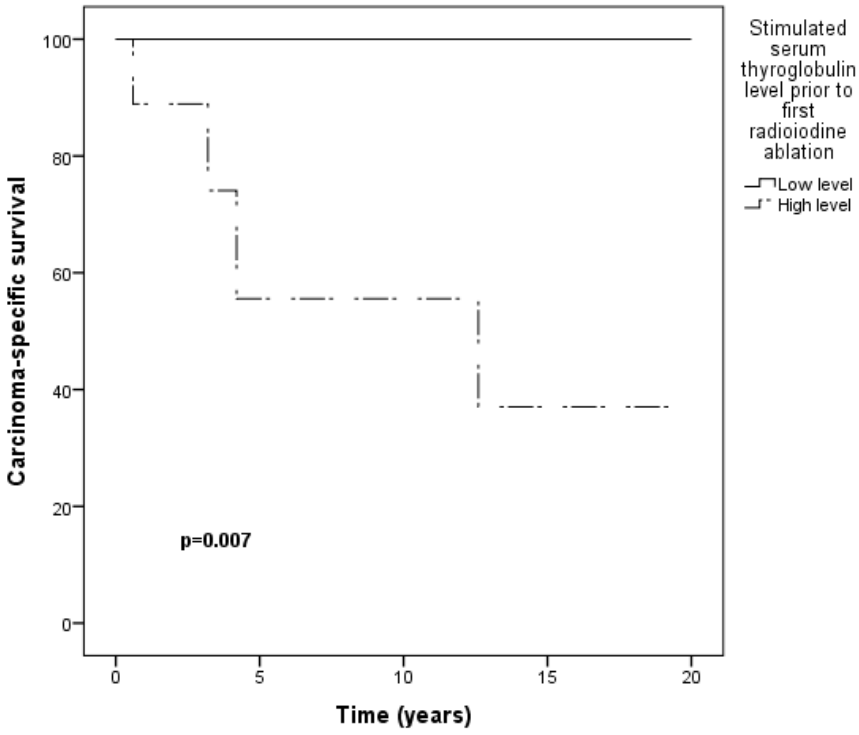


Figure 12. Kaplan-Meier survival figure showing a correlation between high serum thyroglobulin levels and poor survival.

The expression of ERβ was evaluated (III) and a correlation was found with poor prognosis, since each patient suffering a death from disease exhibited a score of ≤5% positive cells in their tumours (p=0.003, IV). Patients with tumours showing ≤5%expression had a 5-year survival of 78%, whereas all patients in the group of high expression were alive at the end of follow-up. Using a cutoff of ≤5% of the ERβ score, nonmetastasized tumours were more often positive than metastasized (either primary or secondary) tumours (p=0.035, unpublished data). No correlation was found between primary and secondary metastasized tumours (p=0.608, unpublished data). Expression of ERβ in WDFTCs was evidently stronger than in PDFTCs, although the difference was not statistically significant, most likely due to the small number of cases (III) (Figure 13).

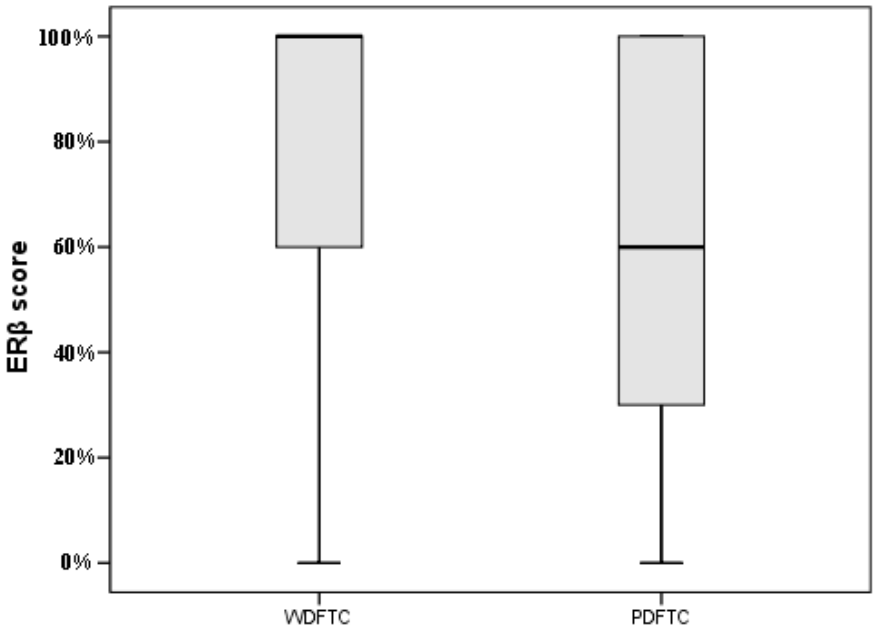


Figure 13. ERβ expression in WDFTC and PDFTC cases in a box plot figure. The whiskers represent extreme values, edges of boxes quartiles and bold line the median value.

It was also found that chronic inflammation (CD45-positive lymphocytes) in tissue adjacent to tumours exhibiting distant metastases was diminished compared with nonmetastasized tumours (p=0.031). In tumours of male FTC patients, chronic inflammation was more diminished in adjacent tissue than in women (p=0.005), while in WDFTCs the correlation was stronger (p=0.002).

9 DISCUSSION

9.2 Clinicopathological features of follicular thyroid neoplasias

Gender and age

The FTC material of the present study consisted of 24 women and 20 men, resulting in a gender ratio of 1.2:1. Other studies showed that thyroid carcinoma, including all subtypes, is 3-fold more common in women than in men (35,36), whereas FTC alone is about 2- to 4-fold more commonly seen in women (49,179,180). In PDFTCs, the gender ratio was, contrasted with that of FTCs, with a male predominance of 1:2.5 (2 women, 5 men). This is in accordance with other studies, since the male gender is often believed to be a poor prognostic trait, with PDTCs previously being classified in other thyroid cancer groups (181,182). However, the number of PDFTCs was small (n=7), thus causing possible bias. The entire adenoma group diagnosed in HUCH region included 287 women and 67 men with a gender ratio 4.3:1, in line with previous results (15).

The reasons for female preponderance in thyroid lesions are unknown. It has been explained by female hormonal and reproductive factors. Pregnancy and early menopause seem to enhance the risk of thyroid tumorigenesis (5,183). It has been speculated that women take better care of their health than men, which leads to earlier discovery of tumours in their benign phase and thus preventing the progression to carcinoma. If a classical multistep tumorigenesis pathway of an FTC arising from an FTA and in some cases even from a goitrous nodule is accepted, this may in part explain why the gender ratio in FTC in the present study was nearly equal instead of showing female preponderance.

In the present study, FTC patients (mean age 58 years) were older than FTA patients (mean age 49 years) at the time of diagnosis. One could speculate that a carcinoma takes a longer time to develop than an adenoma, since carcinomas are believed to evolve from adenomas. The WHO worldwide data on thyroid cancer shows that the FTC incidence peaks in the fifth decade (49), in line with the present study. PTC peaks in the fourth decade of life (40).

Size

In the present study, the carcinomas were on average larger than the adenomas (mean size 4.6 cm versus 2.5 cm, respectively). Again, this is in line with the multistep tumorigenesis of FTC developing from an adenoma in a progressive manner. The WHO showed that a tumour size exceeding a diameter of 4 cm is an adverse prognostic factor for WDFTCs correlating with extrathyroidal growth, multifocality, regional and distant metastases (49,184). A size over 4 cm has correlated with poor prognosis in FTC in previous studies as well (185). All

patients with primary distant metastases (n=7) had a tumour size over 4 cm in diameter, the average size being 7.6 cm, although size did not correlate with the invasiveness of the tumour, nor with metastases, differentiation level of the tumour or survival. In FTC, a tumour size greater than 4 cm (T3 in TNM staging) is associated with lymph node metastasis, an event that is rare in encapsulated FTCs. Lymph node metastases are believed to occur once the tumour has grown enough in size to break through the capsule and spread via the lymphatics (184,186). Evidently, these tumours are widely invasive and thus have a poorer prognosis than smaller carcinomas. In the present study, five patients had nodal invasion at the time of diagnosis, one exhibited a T3 tumour and the rest had a T4 tumour in line with the previous study.

Oxyphilia

The tumour material consisted of 15 oxyphilic FTCs (34% of all FTCs) and of 29 oxyphilic FTAs (8% of all FTAs), showing that oxyphilia is more common in carcinomas. Oxyphilic tumours harbour mutations in mitochondrial DNA, inflicted by high levels of oxidative stress in thyroid cells. This leads to energy production defects and to a production of excess amounts of dysfunctional mitochondria as a compensatory mechanism. Alterations in mitochondrial DNA increase susceptibility to thyroid tumorigenesis (187). Acquisition of an oxyphilic appearance is time-consuming and oxyphilic cell transformation is believed to be a continuous process. This goes in line with observations showing more oxyphilia in FTCs, which are believed to evolve from FTAs, according to the classical multistep carcinogenesis concept. Patients with oxyphilic carcinomas showed similar survival compared with their nonoxyphilic counterparts. Oxyphilia, as well as inflammation, are associated with high levels of oxidative stress. Thus, instead of causing malignant transformation, it may simply be a consequence of oxidative metabolism, since similar survival figures for oxyphilic and nonoxyphilic carcinomas have been shown in other studies as well (187). On the other hand, oxyphilia is associated with a poorer ability to concentrate iodine, which makes oxyphilic tumours less responsive to radioiodine therapy (187).

Poorly differentiated thyroid carcinoma

In 2004, the WHO introduced PDTC, a new entity in the spectrum of thyroid neoplasias (64). Thus, the incidence numbers prior to 2004 are largely unknown. The WHO stated that the incidence of PDTC may be up to 4–7% of diagnosed thyroid carcinomas. Both lower and higher figures have been reported: 1% in Japan, 6% in the United States and 7–13% in Italy (63,130,188,189). Such variance in the incidence numbers is probably caused by the different definitions used, such as diversities shown between WHO definition and Turin proposal, with the latter having more strict criteria (130). Geographical reasons are also considered, due to the high variance between countries; US 1.8% versus Italy 7% (130). The role of iodine deficiency has been suggested as one possible reason (130). In the present study, 16% of

FTCs were PDFTCs (7 of 44 patients). Among PTCs, the prevalence is as low as 1% (63,190). Apparently, it seems that FTC is more prone to dedifferentiate into PDTC than PTC.

9.2 Immunohistochemistry

9.2.1 Non-neoplastic lesions

Several markers have been studied to differentiate between follicular neoplasias and non-neoplastic lesions of the thyroid. Markers such as e-cadherin, syndecan-1, CK-19, galectin-3, HBME-1 and RAS have shown significant correlation, but none show sufficiently high enough values for sensitivity and specificity or adopted a significant role in the clinical diagnostics (58,123,191-193). Clinically, the differentiation between a non-neoplastic and a neoplastic thyroid lesion is important, because recommended management of neoplastic thyroid lesions is surgery, whereas non-neoplastic lesions need only to be kept under surveillance (27). Further research is needed, because thyroid nodules are common in population and the increasing use of modern imaging methods has led to an increasing number of small lesions detected either by purpose or incidentally.

A novel antibody HES5 was studied (II) that had not been published or described previously. HES5 expression differed significantly between non-neoplastic and neoplastic follicular thyroid lesions. Nuclear expression of HES5 was stronger in non-neoplastic lesions, whereas cytoplasmic expression was more marked in neoplastic lesions. Nuclear expression of HES5 more sensitive and spesific than cytopasmic staining in predicting the likelihood of neoplasia. However, the expression showed too low a level of sensitivity and negative predictive values for it to be used alone in differential diagnostics, but as part of a panel of immunohistochemical markers it may be of benefit.

Experiments so far have failed to unravel the antigen of the HES5 antibody against a human embryonic stem cell line. The function of this antigen in the cell and in tumorigenesis of follicular thyroid neoplasias is also unknown, although the findings of this study indicate a role in early tumorigenesis. The findings of this new stem cell marker distinguishing between thyroid non-neoplasia and neoplasia suggest that in addition to the classical multistep model, the more recently presented CSC theory may also play a role in thyroid carcinogenesis.

Interestingly, 4 out of 41 goitrous lesions in the study material showed negative nuclear staining for HES5, which may indicate the neoplastic nature of these lesions, although none showed strong cytoplasmic staining. In a study by Derwahl et al. (194), goitre is believed to be a true neoplasia that arises from a small group of functionally and structurally heterogeneous thyrocytes having a high intrinsic and autonomous growth potential, which secures the proliferation of new follicles and thus the basal secretion of thyroid hormones.

These cells may be affected by the overexpression of an oncogene or a growth factor, leading to formation of a tumour (194). The genetic predisposition of developing nontoxic MNG is linked to a gene locus on chromosome 14q, and this predisposition is also associated with thyroid cancer formation (195). Normal thyroid tissue is polyclonal whereas hyperplastic or goitrous tissue can be both monoclonal and polyclonal, and malignant tissue monoclonal (196-198). Thus clonality of the tissue cannot distinguish between hyperplasia and neoplasia (198). For example thyroid carcinoma is thought to originate as a monoclonal tumour that can progress to polyclonal tumour as secondary genetic alterations occur (74). It has been proposed that an adenoma might evolve from a goitrous nodule trough a hyperplastic process with more rapidly and autonomously proliferating cells leading to somatic mutations and eventually tumour development (199,200).

9.2.2 Neoplastic lesions

Several factors, including goitre, adenoma and inflammation, promote development of thyroid cancer (59,156,158). By understanding the path of thyroid carcinogenesis, the various factors involved have been investigated to find a marker for differentiating between benign and malignant thyroid lesions. In the present study, the markers MIB-1, Cyclin D1, ERβ and TLR-2 differentiated between FTA and FTC.

The MIB-1/Ki-67 and Cyclin D1 markers, indicating the ability of tumours to proliferate and supporting the classical multistep carcinogenesis pathway, were both expressed more strongly in FTCs than in FTAs. Still, the expression of Cyclin D1 showed no significant correlation with clinical parameters or with survival. The most promising markers in differentiating between FTA and FTC in the present study, were MIB-1 and ERβ. Currently, MIB-1 is in clinical use to assess the proliferation of tumour; in thyroid carcinomas it is most often used in evaluation of PDTC and ATC (103-105,113,136). Logistic regression and ROC curve analyses showed that ERβ is stronger as a differential marker between FTA and FTC than MIB-1. On the other hand, ERβ showed too low a level of sensitivity and specificity for being used alone, but it may be exploited as part of a panel of markers. In the present study, the higher expression of ERβ in FTAs than in FTCs suggests that classical multistep pathway is valid in the follicular carcinogenesis of thyroid. The results go in line with ERβ being acknowledged as a tumour suppressor important in maintaining the cell´s differentiated and epithelial phenotype. ERα, which induces cell proliferation and invasiveness of thyroid cancer cells, was unfortunately expressed only weakly, with no significant correlation with clinicopathological characteristics in the present study (140,201-203). If expression of markers, such as ERβ and MIB-1, were evaluated from thyroid cytology specimens, it would lead to higher specificity of FNAB, to earlier and more reliable diagnosis and could save money and time.

TLR-2 and TLR-4, factors involved in inflammatory reactions, were studied (IV). Only TLR-2 differentiated significantly between FTA and FTC, but showed no correlation with major clinicopathological characteristics of tumours.

9.2.3 Aggressive follicular thyroid carcinomas

Although FTC often acts indolently, it is important to distinguish those patients with an aggressive disease as early as possible to direct them to a more thorough management. The markers MIB-1, ERβ, CD45 and TLR-4 showed prognostic value, thereby detecting those patients that might need more aggressive treatment (I, III, IV). No correlation was detected between p53 expression and clinicopathological features, since the majority of all neoplasias studied showed negative or mild expression, even in the PDFTCs (I). In other studies, p53 expression was positive in 20–30% of cancer cells in PDTC (64), whereas in this study material all PDFTCs showed a score below 20% of positive cells.

The results of ERβ and MIB-1 (I, II) support the theory of multistep carcinogenesis, i.e. PDFTC to origin from WDFTC, since significant difference in expressions were shown between the differentiation levels of the tumours. Both markers showed a significant correlation with poor survival. All patients who died from disease exhibited a diminished level of ERβ expression (III). ERβ is believed to protect against hyperproliferation and its loss is linked tumour dedifferentiation and progression. A prognostic association was also detected in breast, ovarian cancer and lung cancers (141-143). The results of the present study suggest the clinical value of MIB-1 and ERβ in preoperative risk management of patients.

Neither ERα or β expressions showed any differences between the genders, suggesting again a unisex purpose for these receptors. An interesting observation was also made, in which male patients´ tumours exhibited fewer inflammatory cells in the adjacent tissue than female patients´ tumours (IV). Thyroid cancer is approximately three times more common in women, but more aggressive cancers are more often encountered in men and the male gender is a well-known risk factor for poor prognosis (181,182), although no prognostic difference was observed between the genders in this study. Thus, one might ponder on whether the female gender is a protective feature in thyroid carcinomas, perhaps explained by differences in estrogen hormone levels, other biological differences or differences in behaviour concerning health issues.

Chronic inflammation is a major factor in human tumorigenesis (150,151). The chronic presence of inflammatory cells, various free radicals, chemokines and growth factors is able to alter normal cell homeostasis, leading to genomic instability to oncogenes and tumour suppressor genes (163,204). On the other hand, the presence of protective immune cells, such as lymphocytes and dendritic cells, is associated with better survival in thyroid cancer (157).

TLRs, important in inflammatory reactions, are considered as a double-edged sword in cancer, with both promoting and inhibiting effects (169). The results concerning TLR-4 expression show that aggressiveness of tumour correlates with a dual staining pattern, either moderate to strong or absent expression. Non-aggressive tumours expressed TLR-4 only mildly in the present study. Indeed, increased expression of TLR-4 has been associated with malignancy, poor differentiation and poor survival in lung, rectal and breast cancer

(205-208), whereas contrasting, i.e. decreased expression is linked to malignancy and poorer outcome in colon and prostate cancer (209,210). Such diverse effects may be caused by a dysfunctional receptor failing to awaken immune reaction against unknown neoplastic cells, but on the other hand lack of expression leads to a failure to recruit immune cells.

9.3 Study limitations

FTC is a rare cancer type. Approximately 350–400 new thyroid cancer cases are diagnosed yearly in Finland, with a population of 5.4 million (31). About 10% of all thyroid cancer cases are FTCs (35–40 cases per year). In the HUCH region, with approximately 1.1 million residents, the approximate number of FTCs diagnosed currently per year is 7–8 new cases. And when the incidence rate of new FTC cases decreasing by an average of 15% in 20 years according to the Finnish Cancer Registry is considered, the cancer material in the present study showed a lower number than suspected. The reasons for this discrepancy may be due to inaccurate categorization, since currently the guideline is to carefully sample the capsule of follicular neoplasias for possible invasions. But inaccurate data given to the Registry, regional differences in diagnostic criteria, as well as surgery performed in the private healthcare sector may also obscure the true number of FTCs in the HUCH region and in Finland.

FTC is an often indolently behaving cancer type; thus not many disease-related deaths are available. Thus, this material is unique with eight disease-related deaths, due to its small size, chance or bias must be acknowledged in interpreting results. It would be of great interest to gather a larger multicenter cohort of FTCs to restudy the antibodies of interest to obtain more reliable and less-biased results.

The immunohistochemistry method used is a remarkably sensitive and specific method used for the past 50 years to study the antigenic expression of cells and tissues, with feasible morphological preservation of histological and cytological samples. The negative aspects of immunohistochemistry include the risk of false-negative results, due to inappropriate antibody, autolysis of antigen, antigen density below the detection capacity, and false-positive results, due to cross-reactivity of the antibody, nonspecific binding, endogenous peroxidase present, entrapment of normal cells inside the tumour and entrapment of proteins of normal cells inside the tumour cells (211).

Whole tissue sections (I, IV) and TMA sections (II, III) were used. Use of whole-tissue sections requires a large quantity of tumour material, thus diminishing the future use of the same material, which is especially unfortunate, considering the rare tumour material of FTCs. Additionally, tumours, especially carcinomas of poor differentiation, often have heterogenic areas of differentiation level. For example, in using the MIB-1 stain, analysis is made from hot spots of the tumour, i.e. the most actively proliferating areas. To obtain

equal proliferation rates, as in whole-tissue sections, stains such as MIB-1 should not be assessed from TMA sections. The TMA procedure is thus mostly valid when antibody is expressed simultaneously throughout the section and the role of intratumour heterogeneity is further diminished by taking specimens from the peripheral and central areas of the tumour. Studies have shown that a strong correlation exists between TMA sections and whole-tissue sections; using large cohorts of tumour material, the reliability of the marker studied is further increased (212). The present study, with only 44 cancers and 83 adenomas, thus includes the risk of bias, due to the small number of cases.

In comparison to whole-section analysis, TMA´s obvious benefit is the feasibility of studying large quantities of tumours in a time-saving and cost-saving manner, and the staining quality is consistent between each spot. On the other hand, TMA is prone to disturbance by the quality of the tissue, which was also evident in this study, since thyroid tissue consists of major amounts of colloid, which easily causes the tissue to only loosely attach to the slide. Excellent technical skills are also required for use of TMA machinery.

9.4 Tumorigenesis of follicular thyroid tumours

The results of the present study showed that at least two major pathways for follicular thyroid tumorigenesis are applicable. A novel stem cell marker HES5 reinforces the role of the stem cell theory in the tumorigenesis of follicular thyroid neoplasia evolving from a non-neoplastic lesion. On the other hand, several markers of the present study support classical multistep tumorigenesis, with an FTC evolving from an FTA and further a PDFTC from a WDFTC. The results showed that these theories do not contrast with each other, and further studies are needed for more detailed results.

10 CONCLUSIONS

The aim was to obtain diagnostic aid in the field of follicular thyroid neoplasia. This material included 44 FTCs (7 PDFTCs), 83 FTAs and 86 non-neoplastic thyroid lesions as a control group.

The major findings were:

1) Clinicopathological re-evaluation
   - ♦ Up to 16% of FTCs showed evidence of poor differentiation in HUCH patients
   - ♦ Oxyphilic tumour cells were more commonly found in carcinomas than adenomas
2) Several demographic features were acknowledged in the present study:
   - ♦ The incidence of FTCs in Finland has been declining since the 1970´s
   - ♦ Female preponderance was more evident in FTAs than FTCs
   - ♦ Patients with FTCs were older and their tumours larger than FTA patients
3) Specific immunohistochemical markers correlated significantly:
   - ♦ HES5 differentiated between non-neoplastic and neoplastic follicular thyroid lesions
     - High nuclear expression in non-neoplastic lesions
     - High cytoplasmic expression in neoplastic lesions
   - ♦ MIB-1, Cyclin D1, TLR-2 and ERβ differentiated between FTA and FTC
     - Higher MIB-1 and Cyclin D1 expressions in FTCs than in FTAs
     - Lower TLR-2 and ERβ expressions in FTCs than in FTAs
   - ♦ TLR-4 differentiated those FTCs of an aggressive nature
     - Both negative and high expressions correlating with aggressiveness
   - ♦ MIB-1 and ERβ had prognostic value in assessing fatal FTCs
     - High MIB-1 and low ERβ expressions correlating with poor prognosis

In conclusion, the markers presented in this thesis can be used as an aid in the diagnosis of follicular thyroid lesions in revealing their neoplastic and malignant nature, as well as pointing to patients with high risk of aggressive and even fatal disease.

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